Reproductive Patterns and Population Genetics in Pure Hybridogenetic Water Frog Populations of *Rana esculenta*

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde (Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Martina Arioli

von

Gurtnellen UR

Promotionskomitee

Prof. Dr. Heinz-Ulrich Reyer (Vorsitz und Leitung)

Prof. Dr. Trevor J.C. Beebee

Prof. Dr. Paul I. Ward

Zürich 2007

Die vorliegende Arbeit wurde von der Mathematisch-naturwissenschaftlichen Fakultät der Universität Zürich am 1. Oktober 2007 als Dissertation angenommen.

CONTENTS

GENERAL INTRODUCTION

Hybridization and clonality

The evolutionary potential of interspecific hybridization has been a controversial issue for quite some time. While seen as widespread and an important process for speciation by botanists (Grant 1981), zoologists usually considered it as a process that limits diversification and therefore dispatched this mechanism as an evolutionary dead end (Mayr 1963). When two species or diverse populations of a species hybridize, the produced F1 hybrids usually have lower fitness than the two parental species. Due to their intermediate morphological and physiological characters, the hybrids are often at a selective disadvantage in either one of the parental habitats. Besides such exogenous selection, endogenous selection against hybrids can arise from dissimilarity of genes causing depressed fertility, developmental instability and hence loss of fitness (Bronson et al. 2003, Peterson et al. 2005).

Yet, hybridization events are common in nature (reviewed by Arnold 1997 and Mallet 2005). It has been hypothesized that the majority of angiosperms is derived from past hybridization events (Grant 1981). In animals the presumed number of natural hybridization events is clearly lower; Grant and Grant (1992) reported in their review that 9 % of the investigated bird species were regularly producing hybrid offspring and Hubbs (1955) found that 3–17% of the investigated fish species were frequently hybridizing. But in both, plants and animals, the occurrence of hybrids is very unequally distributed among genera and taxa (Grant and Grant 1992, Ellstrand et al. 1996, Mallet 2005). This heterogeneity could have several explanations such as biological reasons (mating behavior or ecological distribution), evolutionary causes (early hybridization event with subsequent formation of several closely related species) or simply reflects a sampling bias. Although most of the above mentioned hybridization events lead to inviable or sterile hybrid offspring, many contemporary populations of plants and animals show evidence of hybridization and introgression (Arnold 1997). Some of these formed hybrid taxa even have maintained large populations and existed over long time periods (Hedges et al. 1992, Bullini 1994, Schartl et al. 1995, Alves et al. 2001, Janko et al. 2003). Several models have been put forward to explain the existence and, in some cases, even success of hybrid lineages. The tension zone model postulates that, although hybrids are selected against, hybrid zones are maintained by the continuous new formation of hybrids in an area where the parental species overlap (Barton and Hewitt 1985). Additionally, two other environment-dependent models have been put forward, which allow the

hybrid to coexist with the parental species. The hybrid superiority model (Moore 1977) proposes that hybrids are more fit than either of the parental phenotypes in a hybrid zone which is usually narrow and located in an ecotonal region. At the same time this model also implies that hybrids are less fit in the parental habitats. The mosaic model (Harrison and Rand 1989), in contrast, assumes not an ecotonal hybrid zone, but instead a patchy environment in the contact zone and explains the occurrence of hybrids with a different habitat preference of the hybrid and the two parental species for specific ecological patches. While the tension zone model only allows for ecological success, the hybrid superiority and the mosaic model predict also evolutionary success with the potential for speciation.

If hybrids and their parental species live in close vicinity as proposed in all the models above, there is a possibility of backcrossing. This process allows a species to incorporate foreign alleles and can have different evolutionary consequences. On one hand the genetic variability within a species can be enhanced and, therefore, problems based on low variability, such as inbreeding, can be avoided. But on the other hand the adaptation to a specific habitat can be weakened and negatively influence a species.

As mentioned above a fair number of taxa nowadays are of hybrid origin. So if in fact hybridization plays an important role in speciation the hybrid taxon needs to establish itself separate from the parental species and evolve independently. In intermediate habitats hybrids often have a fitness advantage, because their optimum lies between that of the parental species. Also, in new or disturbed habitats the hybrid's gene combination might be better adapted than that of any of the ancestors. According to Kearney (2005) this offers an explanation why hybrid success is often associated with postglacial habitats, since those habitats are marginal, novel and perturbed.

A precondition for colonizing new areas is not only the invasion, but also the successful reproduction on site. Successful reproduction is particularly challenging for hybrids since they are often sterile or have very low fertility. Often associated with this sterility or low fertility is the difficulty of accurate pairing of homologous chromosomes during gametogenesis. Depending on the amount of dissimilarity between the parental genomes this can lead to serious problems in reproduction. Therefore, it is not surprising that successful hybrid taxa are often characterized by altered reproductive systems that allow overcoming such fertility difficulties. Three such alternative reproductive modes often found in hybrid taxa are parthenogenesis, gynogenesis and hybridogenesis (Dawley 1989). In parthenogenesis the genome is transmitted clonally to the offspring without recombination. Parthenogens are usually

unisexual and theoretically a single female can found a new population. Gynogens are unisexual as well and reproduction is also clonal, but although not incorporated in the zygote, sperm is needed to stimulate development of the egg. Thus, gynogenetic females are still depending on males from one parental species. This is similar to hybridogenesis, where only half of the genome is transmitted clonally to the offspring and the other half of the genome comes from one of the parental sexual species ("hemiclonal reproduction"). For gynogenetic and hybridogenetic animals colonization of new habitats is therefore somewhat more difficult, because they need mates of the ancestor species to produce offspring. They are forced into coexistence with their sexual host and can spread only when the host does so as well.

Clonal and hemiclonal organisms (be they hybrids or not) are often though to be "handicapped" taxa because the only source of variation is mutation, and deleterious mutations accumulating on the clonal genome through Muller's ratchet (Muller 1964) can not be purged through recombination. Hence, the evolutionary potential of these organisms is hotly debated among evolutionary biologists (Judson and Normark 1996, Little and Hebert 1996, Kearney 2005). It can not be denied, however, that hybrid taxa and clonal reproduction are widespread and occur in several genera of vertebrates (reviewed in Vrijenhoek et al. 1989) and that some taxa even have achieved remarkable evolutionary longevity (Vrijenhoek 1994, Judson and Normark 1996). A major advantage of clonality is that adapted gene complexes do not get broken up through recombination and that, due to the fact that they are unisexual, these organisms save the two-fold costs of males (Maynard Smith 1978).

In order to understand why some clonal hybrid taxa have such an ecological and evolutionary success, we need to study the dynamics and structure of such clonal hybrid populations. A system with several hybrid taxa that are successful in terms of geographic distribution, population size and long-term persistence exists in European water frogs. In my PhD thesis, I focus on processes and mechanisms that prevail in an area with only pure hybrid populations in order to understand the hybrid success and its advantage compared to the parental species.

Water frog complex: the hybrid taxon *Rana esculenta*

The hybrid taxon *Rana esculenta* Linnaeus, 1758 (edible frog, genome composition LR) is formed through hybridization between two European water frog species, the lake frog *Rana ridibunda* Pallas, 1771 (genome composition RR) and the pool frog *Rana lessonae* Camerano, 1882 (genome composition LL). There have been several primary hybridization events (Graf and Polls Pelaz 1989, Beebee 1996, Guex et al. 2002), but today the two parental species are seldom found in sympatry due to different ecological requirements (Holenweg Peter et al. 2002). In contrast to most known hybridogenetic systems, such as in the genera *Bacillus* (Mantovani and Scali 1992), *Ambystoma* (Hedges et al. 1992) or *Poeciliopsis* (Vrijenhoek 1994), *R. esculenta* is a bisexual hybrid, i.e. both, males and females, reproduce by hybridogenesis (Schultz 1969). In this mode of reproduction, one genome is clonally transferred to the gametes, while the other genome is discarded before meiosis (Tunner and Heppich-Tunner 1991). Due to its clonal inheritance, the transferred genome has often accumulated deleterious mutations and, consequently, matings between two hybrids produce no viable offspring (Semlitsch and Reyer 1992, Vorburger 2001). Hybrid condition is restored in each generation by backcrossing, i.e. fusion of the clonal gametes of the hybrid with gametes from the sexual parental species whose genome is discarded (Fig.1a). This “hemiclonal” reproduction (Dawley 1989) forces the hybrid into coexistence and mating with at least one of the parental species. In a way, the hybrid parasitizes the parental species by “stealing” and then discarding its genome. However, as in any parasite-host system, the hybrid’s existence depends on the maintenance of the parental species, and stable coexistence is only achieved within certain boundary conditions with respect to mating preferences, female fecundity and larval survival (Hellriegel and Reyer 2000).

This sexual parasitism is reflected by the widespread occurrence of two types of mixed populations: *R. lessonae/R. esculenta* (LE-system) and *R. ridibunda/R. esculenta* (RE-system). But despite this normally necessary sympatry with one of the parental species, all-hybrid populations have arisen, predominantly in the northern region of the distribution, i.e. in Sweden (Ebendal 1979), Denmark (Fog 1994) and Northern Germany (Günther 1975b). In these areas the hybrid has become reproductively independent from the parental forms. Therefore, these pure hybrid populations offer an ideal system to study the ecological and evolutionary success of a hybrid taxon over the parental species.

One of the key factors in the formation of pure hybrid populations of *R. esculenta* is the appearance of polyploid individuals. Polyploidy often results from

malfunctioning gametogenesis in hybrids (Schultz 1969, Dufresne and Hebert 1994) and seems to be another mechanism to overcome difficulties in chromosomal pairing during gametogenesis. In the water frog system two common types of triploid animals have been established, i.e. LLR and LRR (Günther 1975b, Ebendal and Uzzell 1982), and very rarely tetraploid individuals (LLRR) are found (Fog et al. 1997, Borkin et al. 2004). Although triploids are also found in mixed LE- or RE-population systems in Northern Germany (Günther 1975b, Eikhorst 1984), Poland (Rybacki and Berger 2001) and the Ukraine (Borkin et al. 2004), they are essential for the maintenance of all-hybrid populations. Figure 1b shows the assumed gamete production and offspring formation in a pure hybrid population under the assumption of random mating.

Approach to my research questions

This study focuses on pure hybrid populations in an area in Scania (Southern Sweden), which lies at the northern border of the water frog distribution. There, the adult population holds only hybrid genotypes and no parental genotypes (*R. ridibunda* (RR) or *R. lessonae* (LL)) (Jakob 2007). Previous studies have shown that, throughout Europe, hybridogenesis does not always proceed in an identical manner. In Western Europe the hybrid *R. esculenta* (LR) discards the L-genome whereas in Eastern Europe it is the R-genome that gets discarded during gametogenesis, and in some areas neither genome is excluded and both are transmitted to the gametes (Günther et al. 1979, Vinogradov et al. 1991). Therefore, the first goal was to identify which gametes are produced by individuals of the three hybrid genotypes and, hence, which offspring are produced and how long they survive under experimental conditions **(chapter 1)**. This was tested by artificially crossing LR, LLR and LRR females and males in all possible combinations and raising the tadpoles under semi-natural conditions. With this experiment it was possible to determine fertilization success of the different genotypes as well as post-zygotic mechanisms, such as genetic compatibility or differential viability that enhance the dominance of certain hybrid genotypes and cause the absence of parental genotypes. For the predominant system in Western Europe, the *R. lessonae*/*R. esculenta* system, it has been shown that hybrid females have much higher fertility (numbers of eggs). So, a hybrid female contributes more to the next generation than a *R. lessonae* female (Abt 2003), but this is mainly due to size differences. Since gametogenesis in hybrids, especially in

triploid individuals, does not seem trivial (Günther 1975a, Vinogradov et al. 1990), it could be expected that some individuals, or whole genotypes, have problems producing gametes and therefore fail to contribute to the offspring generation.

Earlier investigations in the area of Southern Sweden were assuming that it is an area with pure hybrid populations; however, sample sizes and area were always very restricted and genetic methods not always reliable. For **chapter 2** we therefore sampled adult frogs over a period of three years in several ponds differing in their ecology in order to determine the existing genotypes and population compositions in this area.

Artificial crossing experiments, such as described for chapter 1, yield little information about what actually happens in nature. In experiments, post-zygotic selection pressures are relaxed, due to the benign raising conditions, and prezygotic mechanisms, such as assortative mating or different proportions of genotypes at the pond, are excluded. Therefore, we collected data about the formation and change of larval genotypes in natural ponds **(chapter 3)**. Since adult genotype composition is varying strongly between ponds (chapter 2), we considered it important to sample several ponds comprising the whole spectrum of genotype compositions. Although it was not possible with this approach to directly test if prezygotic mechanisms are actually existent, we could at least conclude from the genotypes occurring among fertilized eggs if such mechanisms operate and if they are preventing the formation of parental genotypes (LL and RR) in nature. By following the change in genotype proportions from eggs through larvae to metamorphs, we could identify whether viability of genotypes is affected by differential post-zygotic selection.

In order to understand the evolutionary future and perspective of these all-hybrid populations it is not only necessary to understand demographic and selective processes but also to investigate the genetic diversity and relationship between these populations. The viability of a population is tightly linked with its genetic diversity. It is widely accepted that low genetic diversity can lead to inbreeding and thus to a population decline (Lande 1988, Amos and Balmford 2001). However, although the inheritance pattern in *R. esculenta* is hemiclonal and genetic variability therefore limited, somatically all these frogs are heterozygote. Additionally, polyploidy enhances genetic variability within an individual. We therefore examined these pure hybrid populations for the existing genetic structure and diversity within and between ponds in relation to ecology in these ponds **(chapter 4)**.

The area of Southern Sweden lies at the northern border of the water frog distribution and nowadays is isolated from continental Europe. Apart from Sweden, all-hybrid populations are known to exist in Denmark and in the coastal region of

Northern Germany. The genetic relationship among these all-hybrid populations and with other populations around the Baltic Sea could yield information about possible colonization scenarios and the origin of pure hybrid populations in the study area. In **chapter 5**, we therefore sampled water frogs from the whole area around the Baltic Sea and investigated their population composition and genetic relationship.

a)	Males	LR	LL
Females	Gametes	R	L
LR	R	RR	LR
LL	L	LR	LL

b)	Males	LR		LLR		LRR	
Females	Gametes	R		L		R	
LR	LR/R	LRR	RR	LLR	LR	LRR	RR
LLR	L	LR		LL		LR	
LRR	R	RR		LR		RR	

Figure 1. Schematic figure showing the production of gametes for females and males for (a) a mixed *R. lessonae*/*R. esculenta* system found in Switzerland and (b) a pure *R. esculenta* hybrid system present in Southern Sweden consisting of diploid LR and triploid LLR and LRR individuals. Shown are the offspring types that are expected from random mating. Genotypes in grey boxes do not occur among the adults in the population although they are produced initially.

References

Abt, G. 2003. Pond use, patterns of reproduction and juvenile recruitment in a mixed waterfrog population. PhD-Thesis. University of Zurich, Switzerland.

Alves, M. J., M. M. Coelho, and M. J. Collares-Pereira. 2001. Evolution in action through hybridisation and polyploidy in an Iberian freshwater fish: a genetic review. Genetica **111**:375-385.

Amos, W., and A. Balmford. 2001. When does conservation genetics matter? Heredity **87**:257-265.

Arnold, M. L. 1997. Natural hybridization and evolution. Oxford University Press, New York, NY.

Barton, N. H., and G. M. Hewitt. 1985. Analysis of hybrid zones. Annual Review of Ecology and Systematics **16**:113-148.

Beebee, T. J. C. 1996. Ecology and conservation of amphibians. Chapman & Hall, London.

Borkin, L. J., A. V. Korshunov, G. A. Lada, S. N. Litvinchuck, J. M. Rosanov, D. A. Shabanov, and A. I. Zinenko. 2004. Mass occurrence of polyploid green frogs (*Rana esculenta* complex) in eastern Ukraine. Russian Journal of Herpetology **11**:203-222.

Bronson, C. L., T. C. J. Grubb, and M. J. Braun. 2003. A test of the endogenous and exogenous selection hypotheses for the maintenance of a narrow avian hybrid zone. Evolution **57**:630-637.

Bullini, L. 1994. Origin and evolution of animal hybrid species. Trends in Ecology and Evolution **9**:422-426.

Dawley, R. M. 1989. An introduction to unisexual vertebrates. Pages 1-18 *in* R. M. Dawley and J. P. Bogart, editors. Evolution and Ecology of Unisexual Vertebrates. New York State Museum, Albany New York, USA.

Dufresne, F., and P. D. N. Hebert. 1994. Hybridization and origins of polyploidy. Proceedings of the Royal Society of London Series B **258**:141-146.

Ebendal, T. 1979. Distribution, morphology and taxonomy of the Swedish green frogs (*Rana esculenta* complex). Mitteilungen aus dem Zoologischen Museum Berlin **55**:143-152.

Ebendal, T., and T. Uzzell. 1982. Ploidy and immunological distance in Swedish water frogs (*Rana esculenta* complex). Amphibia-Reptilia **3**:125-133.

Eikhorst, R. 1984. Untersuchungen zur Verwandtschaft der Grünfrösche: Verbreitung, Struktur und Stabilität von reinen *Rana esculenta*- Populationen. PhD-Thesis. University of Bremen, Germany.

Ellstrand, N. C., R. Whitkus, and L. H. Rieseberg. 1996. Distribution of spontaneous plant hybrids. Proceedings of the National Academy of Sciences of the United States of America **93**:5090-5093.

Fog, K. 1994. Water frogs in Denmark: Population types and biology. Zoologica Poloniae **39**:305-330.

Fog, K., A. Schmedes, and D. Rosenørn de Lasson. 1997. Nordens padder og krybdyr. G.E.C. Gads forlag, Copenhagen.

Graf, J.-D., and M. Polls Pelaz. 1989. Evolutionary genetics of the *Rana esculenta* complex. Pages 289-302 *in* R. M. Dawley and J. P. Bogart, editors. Evolution and Ecology of Unisexual Vertebrates. New York State Museum, Albany, New York, USA.

Grant, P. R., and B. R. Grant. 1992. Hybridization of bird species. Science **256**:193-197.

Grant, V. 1981. Plant speciation. Columbia University Press, New York, NY.

Guex, G. D., H. Hotz, and R. D. Semlitsch. 2002. Deleterious alleles and differential viability in progeny of natural hemiclonal frogs. Evolution **56**:1036-1044.

Günther, R. 1975a. Untersuchungen der Meiose bei Männchen von *Rana ridibunda* Pall., *Rana lessonae* Cam. und der Bastardform "*Rana esculenta*" L. Biologische Zentralbibliothek **94**:277-294.

Günther, R. 1975b. Zum natürlichen Vorkommen und zur Morphologie triploider Teichfrösche, "*Rana esculenta"* ,L., in der DDR (Anura, Ranidae). Mitteilungen aus dem Zoologischen Museum Berlin **51**:145-158.

Günther, R., T. Uzzell, and L. Berger. 1979. Inheritance patterns in triploid *Rana "esculenta"* (Amphibia, Salientia). Mitteilungen aus dem Zoologischen Museum Berlin **55**:35-57.

Harrison, R. G., and D. M. Rand. 1989. Mosaic hybrid zones and the nature of species boundaries. Pages 111-133 *in* D. Otte and J. A. Endler, editors. Speciation and its consequences. Sinauer Associates, Sunderland, Massachusetts.

Hedges, S. B., J. P. Bogart, and L. R. Maxson. 1992. Ancestry of unisexual salamanders. Nature **356**:708-710.

Hellriegel, B., and H.-U. Reyer. 2000. Factors influencing the composition of mixed populations of a hemiclonal hybrid and its sexual host. Journal of Evolutionary Biology **13**:906-918.

Holenweg Peter, A.-K., H.-U. Reyer, and G. Abt-Tietje. 2002. Species and sex ratio differences in mixed populations of hybridogenetic water frogs: The influence of pond features. Ecoscience **9**:1-11.

Hubbs, C. L. 1955. Hybridization between fish species in nature. Systematic Zoology **4**:1-20.

Jakob, C. 2007. Structure and dynamics of pure hybridogenetic water frog populations of *Rana esculenta* in Southern Sweden. PhD-Thesis. University of Zurich, Switzerland.

Janko, K., P. Kotlík, and P. Ráb. 2003. Evolutionary history of asexual hybrid loaches (Cobitis: Teleostei) inferred from phylogenetic analysis of mitochondrial DNA variation. Journal of Evolutionary Biology **16**:1280-1287.

Judson, O. P., and B. B. Normark. 1996. Ancient asexual scandals. Trends in Ecology and Evolution **11**:41-46.

Kearney, M. 2005. Hybridization, glaciation and geographical parthenogenesis. Trends in Ecology and Evolution **20**:495-502.

Lande, R. 1988. Genetics and demography in biological conservation. Science **241**:1455-1460.

Little, T. J., and P. D. N. Hebert. 1996. Ancient asexuals: scandal or artifact? Trends in Ecology and Evolution **11**:296.

Mallet, J. 2005. Hybridization as an invasion of the genome. Trends in Ecology and Evolution **20**:229-237.

Mantovani, B., and V. Scali. 1992. Hybridogenesis and androgenesis in the stick-insect *Bacillus rossius-grandii benazzii* (Insecta Phasmatodea). Evolution **46**:783-796.

Maynard Smith, J. 1978. The evolution of sex. Cambridge University Press, Cambridge.

Mayr, E. 1963. Animal species and evolution. Harvard University Press.

Moore, W. S. 1977. An evaluation of narrow hybrid zones in vertebrates. The Quarterly Review of Biology **52**:263-277.

Muller, H. J. 1964. The relation of recombination to mutational advance. Mutation Research **1**:2-9.

Peterson, M. A., K. J. Monsen, H. Pedersen, T. McFarland, and J. Bearden. 2005. Direct and indirect analysis of the fitness of *Chrysochus* (Coleoptera: Chrysomelidae) hybrids. Biological Journal of the Linnean Society **84**:273-286.

Rybacki, M., and L. Berger. 2001. Types of water frog populations (*Rana esculenta* complex) in Poland. Mitteilungen aus dem Museum für Naturkunde Berlin, Zoologische Reihe **77**:51-57.

Schartl, M., B. Wilde, I. Schlupp, and J. Parzefall. 1995. Evolutionary origin of a parthenoform, the Amazon molly *Poecilia formosa*, on the basis of a molecular genealogy. Evolution **49**:827-835.

Schultz, R. J. 1969. Hybridization, unisexuality, and polyploidy in the teleost *Poeciliopsis* (Poeciliidae) and other vertebrates. The American Naturalist **103**:605-619.

Semlitsch, R. D., and H.-U. Reyer. 1992. Performance of tadpoles from the hybridogenetic *Rana esculenta* complex: Interactions with pond drying and interspecific competition. Evolution **46**:665-676.

Tunner, H. G., and S. Heppich-Tunner. 1991. Genome exclusion and two strategies of chromosome duplication in oogenesis of a hybrid frog. Naturwissenschaften **78**:32-34.

Vinogradov, A. E., L. J. Borkin, R. Guenther, and J. M. Rosanov. 1990. Genome elimination in diploid and triploid *Rana esculenta* males: cytological evidence from DNA flow cytometry. Genome **33**:619-627.

Vinogradov, A. E., L. J. Borkin, R. Günther, and J. M. Rosanov. 1991. Two germ cell lineages with genomes of different species in one and the same animal. Hereditas **114**:245-252.

Vorburger, C. 2001. Fixation of deleterious mutations in clonal lineages: evidence from hybridogenetic frogs. Evolution **55**:2319-2332.

Vrijenhoek, R. C. 1994. Unisexual fish: Model systems for studying ecology and evolution. Annual Review of Ecology and Systematics **25**:71-96.

Vrijenhoek, R. C., R. M. Dawley, C. J. Cole, and J. P. Bogart. 1989. A list of known unisexual vertebrates. Pages 19-24 *in* R. M. Dawley and J. P. Bogart, editors. Evolution and Ecology of Unisexual Vertebrates. New York State Museum, Albany New York, USA.

SUMMARY

Clonal as well as hybrid taxa are, according to evolutionary theory, at a selective disadvantage compared to sexually reproducing organisms and therefore often regarded as evolutionary dead ends. However, there are a surprising number of such “handicapped” taxa that are evolutionarily and ecologically very successful; among them are parthenogenetic reptiles (*Cnemidophorus*), gynogenetic fishes (*Poecilidae*) and hybridogenetic amphibians (*Ranidae*). Several models (i.e. tension zone model, hybrid superiority model or mosaic model) offer an explanation for the success of hybrid taxa beside the parental species. Many of the nowadays recognized species that are of hybrid origin even have shifted from an initial existence alongside its parental species to independence from its ancestors.

The water frog complex offers an excellent system to study the evolutionary and ecological success of a hemiclonal hybrid taxon at the edge of independence. The hybrid *Rana esculenta* was formed by primary hybridization between *Rana ridibunda* and *Rana lessonae*. Due to its hybridogenetic reproduction the hybrid is normally forced into coexistence with at least one of the parental forms and consequently also in competition with the sexual form. Remarkably, however, on the northern border of the distribution we find pure hybrid populations where no parental species are present among the adult frogs. These populations contain not only diploid hybrids but also triploid individuals, which seem to be necessary for the occurrence of these pure hybrid populations. Hence, the aim of this study was to investigate how these pure hybrid populations are maintained and make inferences about their evolutionary perspectives.

In **chapter 1** we investigated with an artificial crossing experiment the gamete production and inheritance patterns of these *R. esculenta* hybrids with particular focus on the triploid individuals. Our data confirmed that triploid males and females produce haploid gametes containing the genome that is present twice in the individual. Both copies of these genomes are passed on in equal proportions and in one female we could demonstrate the occurrence of recombination between these two homologous genomes. Our data showed that diploid females produced mainly diploid eggs resulting in triploid offspring in the next generation. Furthermore, we analyzed the reproductive success of the different hybrid genotypes and showed that genotypes are not significantly differing in their ability to contribute to the next generation. Besides hybrid genotypes (LR, LLR and LRR) also parental genotypes

(LL and RR) were formed among the offspring and under benign rearing conditions they survived at least the first hibernation period. Based on these findings we conclude that the missing parental genotypes among the adults are not exclusively due to lethal genetic problems during their larval development.

For **chapter 2** we sampled over 3 years (2002-2004) adult *R. esculenta* frogs in a variety of ecologically differing ponds in Southern Sweden in order to cover the occurrence of genotypes as well as genotypic population compositions in this area. Our results showed that parental genotypes (LL and RR) are practically absent among the adult individuals. Ponds are usually inhabited by all three hybrid genotypes (LR, LLR and LRR) simultaneously but in different compositions. Additionally, we discovered also viable adult tetraploid individuals. Triploid genotypes were sex biased; LRR individuals were mostly female and LLR individuals were more male than female. Populations were not completely stable over the three sampling years. The proportion of LR frogs increased at the expense of LLR frogs from the first to the third year.

Chapter 3 presents data on the genotype composition during larval development in natural ponds. We collected egg masses, tadpoles and metamorphs at eleven different ponds in Scania and determined their genotypes through molecular methods. Among the egg masses we found that half of the offspring had either a parental genotype (LL, LLL, RR, or RRR), was tetraploid or a genetic mosaic individual. This proportion decreased substantially during larval development up to metamorphosis, when no more parental genotypes were found in our sample. These findings support the hypothesis that mating is random in these ponds and that natural selection acts against the non-hybrid genotypes that are formed, so that the adult population consists of only LR, LLR and LRR animals.

In **chapter 4** we investigated the genetic diversity and genetic population structure of these pure hybrid populations in Scania with regard to their long-term viability. For that we developed microsatellites and examined 33 different populations. Overall genetic variability within the separate genomes (L or R) was very low, but maybe not very surprising considering the northern edge distribution of these populations. Yet, due to its somatic hybrid status, *R. esculenta* is not suffering from direct negative effects resulting from low genetic variability within the discrete genomes. Our data showed that the genetic diversity within the area decreased from a core area to the periphery of the distribution, indicating that the distribution once used to be smaller and that *R. esculenta* is expanding its range again. The population structure is best explained by an “isolation by distance” pattern which

needed enough time to evolve. The ecological characteristics of the pond do not seem to influence the genetic structure of these populations.

In **chapter 5** we examined the relationship between these all-hybrid populations in Southern Sweden and other populations around the Baltic Sea in order to learn more about their evolutionary history. We confirmed the low genetic diversity of the Swedish populations compared with Central Europe, but no straightforward colonization scenario for this region was possible to determine. We assume that during the time when these frogs have reached the area, the basin of the Baltic Sea was not filled with water yet, and frogs were able to migrate between countries. Nevertheless, we found an endemic haplotype among individuals in Southern Sweden as well as in a new found population in Östergötland, indicating that Sweden has been isolated for some time.

ZUSAMMENFASSUNG

Sowohl klonale Organismen als auch Hybride haben laut der Evolutionstheorie einen selektiven Nachteil verglichen mit sexuell reproduzierenden Organismen und werden deshalb oft also evolutionäre Sackgassen angesehen. Es gibt aber eine erstaunlich große Anzahl von solchen „benachteiligten“ Organismen, die evolutionär und ökologisch gesehen sehr erfolgreich sind. Darunter befinden sich parthenogenetische Reptilien (*Cnemidophorus*), gynogenetische Fische (*Poecilidae*) und hybridogenetische Amphibien (*Ranidae*). Verschiedene Modelle, wie z.B. das Tension Zone-Modell, das Hybrid Superiority-Modell oder das Mosaik-Modell, können den gleichzeitigen Erfolg von Hybriden und den Elternarten erklären. Viele der heute als Art betrachteten Organismen, die einen Hybrid-Ursprung besitzen, bestehen nicht nur gleichzeitig neben ihren Elternarten, sondern haben sich auch unabhängig von ihnen etablieren können.

Der Wasserfroschkomplex ist ein ideales System, um den evolutionären und ökologischen Erfolg von solchen Hybriden zu studieren, die auf dem Weg zur Unabhängigkeit von ihren Vorläufern sind. Der Hybrid *Rana esculenta* entstand durch Primärhybridisierung zwischen *Rana ridibunda* und *Rana lessonae*. Durch seine spezielle Fortpflanzungsart (Hybridogenese) ist er normalerweise zur Koexistenz und gleichzeitig Konkurrenz mit mindestens einer der Elternarten gezwungen. Bemerkenswert ist jedoch, dass es am nördlichen Rand des Verbreitungsgebiets von *R. esculenta* reine Hybridpopulationen gibt, in denen keine adulten Elternarten mehr vorkommen. In diesen Populationen findet man neben den gewöhnlichen diploiden Hybriden auch noch triploide Hybride, welche notwendig für die Entstehung von solchen reinen Hybridpopulationen scheinen. Das Ziel dieser Dissertation war es zu untersuchen, wie diese reinen Hybridpopulationen funktionieren und aufrechterhalten werden, sowie Folgerungen bezüglichen ihrer evolutionären Perspektive zu ziehen.

In **Kapitel 1** untersuchten wir mit Hilfe eines künstlichen Kreuzungsexperiments die Gametenproduktion und Vererbungsmuster der Hybriden mit speziellem Fokus auf die triploiden Tiere. Unsere Daten bestätigten, dass triploide Männchen und Weibchen haploide Gameten produzieren, welche das im Tier doppelt vorhandene Genom enthalten. Beide Kopien dieses Genoms werden zu gleichen Teilen vererbt, und in einem Weibchen konnten wir sogar Rekombination zwischen den beiden Kopien dieses Genoms nachweisen. Unsere Daten zeigten, dass die diploiden

Weibchen hauptsächlich diploide Eier produzieren, aus welchen sich triploide Nachkommen entwickeln. Des Weitern untersuchten wir den Reproduktionserfolg von diploiden und triploiden Hybriden und konnten zeigen, dass sie sich darin nicht signifikant unterscheiden. Unter den Nachkommen waren neben den Hybrid-genotypen (LR, LLR und LRR) auch Elterngenotypen (LL und RR) zu finden. Diese überlebten unter guten Aufzuchtbedingungen im Experiment sogar bis nach der Überwinterung. Basierend auf diesen Resultaten kann also ausgeschlossen werden, dass das Fehlen von Elterngenotypen nur auf letale, genetische Probleme während der larvalen Phase zurück zu führen ist.

Für **Kapitel 2** haben wir über 3 Jahre (2002-2004) adulte *R. esculenta* Frösche an verschiedenen Teichen, welche sich ökologisch unterschieden, gesammelt und bestimmt. Daraus ergab sich ein guter Überblick über die Genotypen und Populationssysteme, die Südschweden vorkommen. Unsere Resultate zeigten, dass erwachsene Tiere der Elternarten praktisch nicht vorkommen. Die Teiche bestehen meist aus allen drei Hybridgenotypen (LR, LLR und LRR) welche gleichzeitig, aber in verschiedenen Zusammensetzungen vorkommen. Zusätzlich entdeckten wir auch vereinzelt tetraploide Individuen. Die triploiden Tiere wiesen verschiedene Geschlechter-Verhältnisse auf; LRR Tieren waren meistens weiblich, bei den LLR Tieren fanden sich häufiger Männchen als Weibchen. Die Populations-zusammensetzung der Teiche war über die 3 Jahre nicht stabil; der Anteil von LR Fröschen nahm zu, während der Anteil LLR Frösche zurückging.

Kapitel 3 beschreibt, wie sich die Genotypzusammensetzung in natürlichen Teichen während der larvalen Entwicklung verändert. Wir sammelten Laichballen, Kaulquappen und Metamorphe an elf verschiedenen Teichen in Skåne und bestimmten die Genotypen mit Hilfe von molekularen Methoden. Im ersten Stadium (Laich) hatten etwa die Hälfte der Tiere entweder einen Elterngenotypen (LL, LLL, RR, RRR), waren tetraploid oder genetisch gesehen Mosaiktiere. Dieser Anteil verringerte sich wesentlich im Verlauf der larvalen Entwicklung. Im letzten Stadium (Metamorphe) konnten keine Elternarten mehr gefunden werden. Dieses Resultat unterstützt die Annahme, dass Paarungen am Teich zufällig sind und dass Tiere mit einem Elterngenotypen durch natürliche Selektion aus der Population verschwinden, so dass es nur noch Hybride unter den erwachsenen Fröschen gibt.

In **Kapitel 4** untersuchten wir die genetischen Diversität innerhalb und die genetischen Populationsstruktur zwischen den reinen Hybridpopulationen in Skåne in Bezug auf ihre längerfristige evolutive Zukunft. Aus diesem Grund entwickelten wir Mikrosatelliten und untersuchten 33 verschiedene Populationen aus diesem Gebiet. Die Gesamtvariabilität innerhalb jeder der beiden Genome (L und R) war sehr tief,

was aber nicht überrascht, wenn man bedenkt, dass diese Populationen am nördlichen Rand des Verbreitungsgebiets liegen. Dadurch dass *R. esculenta* somatisch gesehen ein Hybrid ist, hat diese tiefe genetische Variabilität innerhalb der beiden verschiedenen Genome jedoch keinen direkten negativen Einfluss. Unsere Daten zeigten eine Abnahme der genetischen Diversität innerhalb des Gebiets vom Kerngebiet gegen die Peripherie. Dies könnte ein Hinweis dafür sein, dass das Verbreitungsgebiet in Schweden früher kleiner war und sich *R. esculenta* in Skåne wieder ausbreitet. Die Populationsstruktur wird am besten durch ein „Isolation durch Distanz“ - Muster beschrieben, welches eine gewisse evolutive Zeit braucht, um sich entwickeln zu können. Ökologische Teicheigenschaften scheinen dabei keinen Einfluss auf die genetische Struktur der Populationen in diesem Gebiet zu haben.

In **Kapitel 5** geht es um den Zusammenhang zwischen den reinen Hybrid-populationen in Südschweden und anderen Wasserfroschpopulationen rund um die Ostsee. Die geringere genetische Variabilität in Südschweden verglichen mit Zentraleuropa konnte bestätigt werden, doch aus den Daten geht kein klares Kolonisationsszenario für diese Region hervor. Da die Ostsee zu der Zeit, als die Wasserfrösche die Region erreicht haben, teilweise noch trocken war, vermuten wir, dass sich die Frösche damals ungehindert zwischen den untersuchten Gebieten bewegen konnten. Trotzdem fanden wir endemische Haplotypen sowohl in Südschweden als auch in Östergötland als Hinweis dafür, dass diese Gebiete seit einiger Zeit isoliert sind.

CHAPTER 1

Genome inheritance patterns and offspring genotypes in edible frogs (*Rana esculenta*) from pure hybrid populations: First indication for recombination in triploids

MARTINA ARIOLI & CHRISTIAN JAKOB

Abstract.-Hybridization between species is often seen as an evolutionary dead end by zoologists, since hybrids usually have lower fitness than their parental species. Nevertheless, there are some hybrid taxa that are ecologically and evolutionarily very successful. One of them is the water frog *Rana esculenta* (genotype LR), a hybrid between *R. ridibunda* (RR) and *R. lessonae* (LL). Due to its unusual reproductive mode termed hybridogenesis, this hybrid taxon typically occurs in sympatry with one of the parental species. However, at the northern border of the distribution range there exist pure hybrid populations (i.e. without adults of the parental species) which consist of diploid and triploid individuals. With a crossing experiment we investigated the inheritance patterns of these hybrids, their reproductive success and the survival of the formed offspring. Our experiment confirmed the assumed formation of gametes for these Swedish pure hybrid populations. We showed with microsatellite markers that in triploid individuals (LLR and LRR) the genome which occurs twice is inherited by the offspring and that both genome copies are passed on in equal proportions. In at least one triploid individual (female LRR) we demonstrated the occurrence of true recombination. Our data indicate that the different adult genotypes contribute equally to the next generation under the assumption of random mating, since we found no significant influence of genotype on fertilization success, hatchling survival and normal tadpole development. Early developmental success of offspring in the experiment was overall rather low. Parental genotypes (LL and RR) were formed among the offspring in the experiment and, in contrast to our expectations, they survived at least the first hibernation period under benign raising conditions. Therefore, the absence of parental genotypes in the adult population can not be explained by the early death of these genotypes due to genetic problems.

Introduction

The widely used definition of a species implies that it is reproductively isolated from any other group of individuals and that no exchange of genetic material occurs (Mayr 1942). In nature, however, we often encounter the phenomenon of hybridization, where individuals from two distinct populations or even species mate and produce offspring. Since the two parental species are usually to a greater or lesser extent genetically differentiated, F1 hybrids are often sterile or not viable. Hence, endogenous selection acts upon them and prevents successful hybridization. In cases where problems imposed by genetic differentiation are overcome and viable fertile hybrids are formed, they are often still at a selective disadvantage due to environmental factors (Barton 2001). The findings that hybrids have lower fitness than any of the parental species have prompted many zoologists to believe that hybridization is usually a dead end and, therefore, unimportant for evolution (Barton and Hewitt 1985). But recently it has been argued that not all hybrid offspring necessarily have lower fitness (Arnold and Hodges 1995) and some may even perform better than the parental species (Barton 2001). Throughout the last two decades several systems have been described in which hybrids are surviving fairly well and can coexist with their parental species. Examples come from fishes (Quattro et al. 1992, Vrijenhoek 1994, Alves et al. 2001), salamanders (Hedges et al. 1992) and stick insects (Mantovani and Scali 1992).

One of the systems in which the hybrid taxa are very successful is the Palearctic water frog complex. This complex consists of several species which can form different viable and fertile hybrid offspring. The most common and most widely distributed hybrid in Central Europe is the edible frog, *Rana esculenta* Linnaeus, 1758 (genotype LR), which derives from a mating between the pool frog, *R. lessonae* Camerano, 1882 (genotype LL) and the lake frog *R. ridibunda* Pallas, 1771 (genotype RR). The hybrid status of the edible frog was first discovered by Berger (1967). Later, Tunner (1974) showed that the genetic inheritance pattern in these organisms is hemiclonal, i.e. they reproduce via hybridogenesis (Schultz 1969). During gametogenesis, one of the parental genomes is eliminated from the germline, resulting in only one genome being transmitted clonally to the gametes. A new hybrid is formed when this individual mates with the parental species whose genome was eliminated previously (Fig. 1). When hybrids mate with each other, the resulting offspring usually do not survive to metamorphosis, presumably due to the accumulation of deleterious mutations through Muller's ratchet (Semlitsch and Reyer 1992, Vorburger 2001). For the continuity of the hybrid population it is therefore

necessary to coexist and mate with at least one parental species, with *R. lessonae* when the L-genome is discarded and with *R. ridibunda* when the R-genome is eliminated.

However, at the northern edge of the water frog distribution, we find pure *R. esculenta* hybrid populations. These populations without adults of the parental species are remarkable, because mating can only occur between two hybrids. The existence of such pure hybrid populations seems always to go hand in hand with diploid frogs (LR) co-occurring with triploid individuals (genotype LLR and LRR). Fig. 2 shows the assumed production of gametes for the different adult genotypes, the possible mating combinations and the expected offspring genotypes (reviewed by Graf and Polls Pelaz 1989, Günther 1990). Based on this scheme, we expect individuals of the parental species to emerge in the populations, but they are not found among the adult frogs (Jakob 2007 & chapter 2). This raises two questions: First, are the parental genotypes not formed at all, because the respective matings do not occur? Second, is the pattern that is usually found in nature, namely selective superiority of parentals over hybrids, reversed in these populations and are parentals selected against during early larval stages, perhaps due to genetic incompatibilities? Whatever the answers, the absence of reproductive parentals raises a third question: how can these pure hybrid populations maintain themselves?

We performed a crossing experiment using artificial fertilizations to test if the traditional assumptions about the gamete and offspring production hold true for pure hybrid populations in Southern Sweden. We were particularly interested in inheritance patterns in triploid frogs and possible recombination events, because these are crucial for the amount of genetic variation. To investigate the importance of such variation for offspring viability, we also outcrossed Swedish with Polish frogs. We tracked the survival success of the offspring genotypes during the larval and early post-metamorphic stages of their life to see if and when selection acts on them. Since in Swiss *Lessonae/Esculenta* populations different adult genotypes contribute unequally to the next generation (Abt 2003, Reyer et al. 2003), we also compared the reproductive success of the different hybrid genotypes in our experiment.

Methods

Source populations

The study area was located in Scania, Southern Sweden, where native hybrid frogs (*R. esculenta*) were caught during the night at three different source ponds. Two ponds were in close vicinity to each other, the third pond was located about 3 km away. A total number of 88 frogs was collected between May 12 and May 18, 2002 and kept at Stensoffa, the field station of the University of Lund, at 10°C prior to the crossing. Morphological measurements (snout-vent length, tibia length and callus length) and a blood sample from each individual were taken. The blood was analyzed by flow cytometry and results, together with the morphological measurements, were used to preliminarily determine the ploidy and genome composition of these frogs (Jakob 2007). To test for possible outcrossing or inbreeding effects we also included twelve Polish water frogs (from Luszkowo and Rogaczewo) in the experiment (Fig. 3).

Crossing design

Of the 88 Swedish frogs that were caught, twelve females and ten males were used for the crossing experiment. Since not all females are reproducing each year (Reyer et al. 2004), we picked only females for the experiment that were obviously carrying eggs. Each female genotype (LR, LLR, LRR) was mated with each male genotype, and vice versa, using artificial crossings (Berger et al. 1994). For each possible female x male genotype combination we originally planned four replicates, but the actual number of crossings conducted within a “crossing type”, depended on the number of eggs or sperm that were available. Due to the fact that the genotype determination is difficult and was only preliminary at the time when the crossings were done, two frogs turned out to be assigned incorrectly after the final analysis and the design was therefore unbalanced. Specifically, two males that previously had been assumed to be of the genotype LRR turned out to be LLR. In fact, LRR males could not be obtained for the crossing, since they are very rare in this area (Jakob 2007 & chapter 2); none of the 35 captured males had this genotype. A total of 54 crossings (27 crossings with both parents from Sweden, 27 crossings with one Polish parent) were carried out and included in the analysis.

On May 25, one day before performing the artificial crossing, 16 females (12 Swedish frogs and 4 Polish frogs) were injected with a salmon Luteinizing Hormone-Releasing Hormone (LH-RH; H-7525, Bachem), which induces ovulation (Mc Creery and Licht 1983, Licht et al. 1987). The following day, the 18 males (10 Swedish

males and 8 Polish males) were euthanized with ethyl 3-aminobenzoate methanesulfonate (MS-222; A5040, Sigma) and dissected. Their testes were removed and stored in Holtfreter's solution at 4℃ until the crossings were done. Eggs from each individual female were stripped into several Petri dishes filled with filtered pond water. Crossings were performed by adding to each Petri dish a sperm suspension from one male which was sufficient to fertilize all eggs. The newly fertilized eggs were then covered with filtered pond water to ensure the best possible conditions for development. All crossings were done on the same day (day x = May 26). We determined fertilization success per cross as the proportion of eggs in the Petri dish that had rotated their black animal hemisphere to the top (Berger et al. 1994, Reyer et al. 2003).

Rearing design

One day after fertilization (x + 1), the eggs were transferred from Petri dishes into 1–litre tubs containing aged tap water and stored in a lab room at approximately 20℃. The tubs were checked regularly, and unfertilized eggs or embryos that had stopped development were removed in order to maintain a good water quality. The larvae were kept indoors until 12 days after fertilization. On June 7 (x + 12), we counted the number of tadpoles that had hatched. Hatchling survival was determined as the number of hatched larvae relative to the number of fertilized eggs. At this point in time, we also discriminated between tadpoles that appeared to have developed normally and those that showed obvious developmental abnormalities, such as curved, bent or shortened tails, asymmetric or inflated bodies and narrow or thickened heads (Ogielska 1994). The genetic background of these abnormalities and their effects on survival are not yet fully understood. The proportion of normal tadpoles was calculated to investigate if offspring of a crossing exhibit genetical problems which might not be instantly lethal, but handicap the tadpole. On the same day, 15 randomly chosen normal tadpoles from each cross that had produced more than 30 normal looking tadpoles were transferred to 50–litre outdoor tubs. These tubs had been filled 6 weeks earlier with water, inoculated with phyto- and zooplankton and provided with 1-3 snails (*Lymnaea sp*.) to create a self-sustaining aquatic community. The tubs were covered with lids, preventing colonization by invertebrate predators. Each cross was represented by two outdoor replicates (each with 15 tadpoles) which were arranged in a random design. Larval survival rate for these crosses was determined as the survival of tadpoles until July 31 (x + 56) when the experiment was transferred to Switzerland. Many studies have shown that larval growth is not linear (Alford and Jackson 1993, Thurnheer 1999, Vorburger 2001), we

therefore used an exponential growth model (Alford and Harris 1988) to fit the growth pattern of our tadpoles. Growth rate was considered to be the proportional weight gained per day during the time the tadpoles were raised in outdoor tubs in Sweden.

Twenty-eight of the original 54 crosses were either poorly fertilized or mostly abnormally developed by June 7. Tadpoles from those crosses were not transferred to outdoor tubs and instead raised indoors to facilitate the monitoring of their development. Most of these crosses consisted of fewer than 15 tadpoles. We checked these crosses on a daily basis and collected dead individuals for genotype analyses. Most of the tadpoles raised indoors died during their larval stage. Indoor tadpoles that survived until July 31 were euthanized with 3-aminobenzoic acid ethyl ester (MS-222) and frozen for further genotype analysis. From each cross that was raised outside, 10 randomly chosen tadpoles were transported to Zurich, Switzerland, where we monitored their survival and development until metamorphosis was completed. We defined the beginning of metamorphosis as the emergence of at least one forelimb (stage 42, according to Gosner 1960). At stage 42, we removed some tail tissue for later analysis of the genotype and transferred the metamorphs to the laboratory, where they were held in individual plastic containers at 20℃ and checked for survival daily. When tail reso rption was complete (stage 45, Gosner 1960), metamorphs were weighed and the developmental time was noted. This was defined as the time of metamorphosis completion. After metamorphosis, the froglets were raised in indoor terraria, provided with *ad libitum* food (crickets) and checked regularly until early December, when all the survivors were transferred to a cold room (4-5℃), where they hibernated until end of March 2003. Post-larval survival until after first hibernation was calculated as the number of individuals that survived the first hibernation period.

Gamete production of the different genotypes

Parental and offspring genotypes were determined by flow cytometry and microsatellite analysis (Jakob 2007). Thus, we could assess, which gametes had been produced by the different parents. Additionally, all 14 Swedish triploid frogs used in the experiment were tested with 8 microsatellite primer pairs (described in Jakob 2007 & chapter 4) to find triploid animals that were heterozygous at the genome they carry twice in order to examine their inheritance patterns. Six individuals were heterozygous at one microsatellite locus (single heterozygous) and two frogs showed different alleles at two microsatellite loci simultaneously (double heterozygous). One of the single heterozygous individuals, a LRR female, produced only very few ova and we therefore could not calculate a reliable allele inheritance

frequency for this female. From the remaining seven individuals we analyzed between 48 and 166 offspring, including larvae that had developed abnormally, to investigate inheritance patterns. For each individual we calculated the relative inheritance frequency of the larger allele and tested if it differed significantly from the 0.5 expected under random segregation (one-sample t-test). For the two double heterozygous individuals we investigated the proportions of the allele combinations in the offspring.

Statistical analysis

We first tested with an analysis of variance (ANOVA) using PROC ANOVA (SAS Institute 2002-2003) the differences in fertilization success and hatchling survival as well as the proportion of normally developed tadpoles between offspring sired by two Swedish parents (Swe/Swe) and offspring for which either the female (Pol/Swe) or the male (Swe/Pol) was Polish. Pairwise differences were analyzed with a Bonferroni t-test.

In the subsequent analyses we were only interested in the offspring that had two Swedish parents in order to understand how the pure hybrid system in Sweden works. The three early developmental variables (fertilization success, hatchling survival and proportion of normal tadpoles) were analyzed with mixed-model nested ANOVAs using PROC GLM (SAS Institute 2002-2003) to test for the effects of female genotype, male genotype and their interaction as well as for the effects of individual females and individual males nested within genotype. Genotypes were considered fixed effects, but individuals were chosen randomly within the genotypes and therefore considered random effects. The experiment was unbalanced due to problems with correct genotype determination before the crossing and a strong female-biased sex ratio in LRR (see above); we therefore used Type III sums of squares. A RANDOM statement was used to generate mixed-model expected mean squares and error estimates for F-tests using Satterthwaite's approximation. Fertilization success, hatchling survival and proportion of normal developed tadpoles were measured as proportions and therefore arcsine-square root transformed prior to analyses (Stahel 1995). In a one-way ANOVA we tested the effect of offspring genotype on larval survival rate, larval growth rate, weight at metamorphosis, time to metamorphosis as well as post-larval survival rate after first hibernation. The two survival rates were measured as proportions and therefore arcsine-square root transformed before analyses. In order to identify sensitive life stages for the different genotypes, post-larval survival was further divided into survival during

metamorphosis (stage 42 to stage 45), survival as froglet until hibernation and survival during hibernation.

Results

(1) Gamete production

From the genotypes of the offspring we inferred what gametes the parents had produced. Irrespective of their gender, triploid individuals (LLR or LRR) produced haploid gametes containing the genome that was present in two copies (see Fig. 2). In order to test if both genomes get passed on we analyzed individuals that had two different alleles in these two genome copies. For the genotype LLR we had three males and one female that fulfilled this condition. For the genotype LRR, three heterozygous females had been included in the experiment. In all but one male (M1; one-sample *t*-test, $F = 4.68$, $P = 0.043$), the inheritance frequency was not significantly deviant from 0.5 (all $P \geq 0.318$, Fig. 4a). In only one frog (M3) we found that the abnormal offspring from these individuals inherited one of the alleles clearly more often (df = 1, $\chi^2 = 4.83$, $P = 0.049$) than the normal offspring (Fig. 4b). Two of the individuals used in the experiment were double heterozygote and we could therefore test if alleles were inherited together or freely, i.e. recombined. The result was somewhat ambiguous: for the male we found that the same alleles were always inherited together, whereas in the female all four possible combinations occurred in Mendelian proportions (0.25) (Fig. 5). The four diploid females (LR) produced exclusively either diploid LR eggs (3 females) or exclusively haploid R eggs (one female).

(2) Outcrossing with Polish individuals

On average, the mean fertilization rate per cross was 0.537. We found that eggs from Polish females (Pol/Swe) were significantly worse fertilized than those from Swedish females crossed with either Swedish (Swe/Swe) or Polish males (Swe/Pol) (Table 1, Fig. 6a). However, hatchling survival was not significantly influenced by outcrossing and, on average, a proportion of 0.517 of the fertilized eggs developed into tadpoles (Fig. 6b). Overall, a mean proportion of just 0.292 of the eggs turned into normal looking tadpoles, with least of them emerging from crosses with Polish mothers (Fig. 6c).

(3) Larval survival, larval growth and metamorphic traits of offspring genotypes

In line with the gamete production of the parents the following offspring genotypes were found: LR, LLR, LRR, LL and RR (see Fig. 2). Overall, larval survival until metamorphosis was very high (92%) and not significantly different among offspring genotypes (F = 2.98, P = 0.080), although the genotype LL tended to survive slightly worse (75%) than the other genotypes. Time to metamorphosis and weight at metamorphosis, however, differed significantly. The two parental genotypes (LL and RR) were lighter at metamorphosis than the three hybrid genotypes, and it also took the parental genotypes longer to complete metamorphosis (Table 2, Fig. 7). Although not significant, the same picture holds true for daily growth rate that was measured during larval development; compared with the three hybrid types, growth rate of parental genotypes tends to be reduced for both LL and RR (Table 2, Fig. 8).

(4) Post-larval survival of offspring genotypes

Overall post-larval survival rate until after first hibernation was low (29%), but not significantly different for the different offspring genotypes (F = 0.50, P = 0.736). However, survival of the RR genotype tended to be slightly decreased compared to the others (Fig. 9a). When investigating the mortality during the different post-larval stages, there appear to be some differences between genotypes. However, sample size was too low to find significant differences, except between the two triploid genotypes LLR and LRR (df = 2, χ^2 = 7.83, P = 0.015). Most LLR offspring died during hibernation, whereas many of the LRR offspring died before hibernation (Fig. 9b).

(5) Development in relation to parent genotypes

The genotypes of females and males had no significant effects on the early developmental variables, with the only exception that fertilization rate depended on the female x male interaction (Table 3, Fig. 10). For LLR males the fertilization rate was similar, independent of the female type, whereas for LR males the fertilization rate was highest with LR females, intermediate with LLR females and lowest with LRR females. There were, however, clear differences between individuals, in both females and males. This is true for all three developmental variables, although in females individual differences in hatchling survival did not quite reach significance. Independent of the genotype, some males and females had very low and others very high success. The proportion of normal developing tadpoles, for instance, ranged from 0.013 up to 0.701 for females and from 0.002 up to 0.717 for males.

Discussion

In our experiment we showed that it is not early genetic incompatibility that prevents the formation of parental genotypes (LL and RR) in pure hybrid populations of *R. esculenta* consisting of diploid (LR) and triploid (LLR and LRR) frogs. Under benign rearing conditions some individuals with the parental genotypes (LL and RR) survived at least the first hibernation period.

The production of gametes we have found in our experiment agrees with what had been assumed until now (Graf and Polls Pelaz 1989, Fig. 2). In the late 1970s, the observation that diploid hybrid females produced eggs with different size classes led to the suggestion that these eggs have different genome compositions and that females can produce different proportions of haploid/diploid eggs (Berger and Roguski 1978, Günther et al. 1979, Graf and Polls Pelaz 1989, Fog et al. 1997). But as yet it is not clear whether and how these proportions vary between females and/or even within females among years. Three of the four diploid females in our experiment produced only diploid eggs and one produced only haploid R eggs. In pure hybrid populations the reproductive fitness of a diploid female can vary strongly with the ratio of haploid versus diploid eggs. Diploid eggs usually develop into viable triploid frogs; haploid R eggs on the other hand, if fused with R sperm, develop into RR genotypes which are, at some point, eliminated from the adult population and, thus, represent a dead end for their genes. Hence, if the possibility of fusing with an R sperm is high (e.g. in populations with high proportions of LR or LRR males), it pays off to produce mainly diploid LR eggs, as we have found in three of our four animals. But the question remains if the production of haploid versus diploid eggs is determined genetically or environmentally or could even be influenced by the female itself.

Vinogradov et al. (1990) showed that in triploids the single copy genome gets discarded. They assumed that the two remaining genomes are recombined and passed on, but could not provide solid evidence for their assumption, due to the lack of high resolution markers at that time. Using microsatellite markers on a large number of offspring, we could (to our knowledge for the first time) conclusively show that in triploids, LLR individuals transmit both L- and LRR individuals both R-genomes. Except in one male, both copies of the transferred genomes were passed on with an approximate proportion of 0.5, as expected under random chromosome segregation (Fig. 4a). The occurrence of abnormalities during development does not seem to be associated with just one of the genomes, because in most cases the normal and abnormal tadpoles did not differ in which genomes they inherited (Fig.

4b). But, as mentioned before, developmental abnormalities can be manifold and probably have to be explained by the interaction of genetical problems with environmental influences such as infections, toxins etc. (Ogielska 1994, Guex et al. 2001).

Regarding the question of recombination, our results are not conclusive. Unfortunately, we had only two triploid animals which were double heterozygous for the genome they possessed twice. This is not surprising, because we found very low genetic variation in these Swedish populations (chapter 4). Offspring of the double heterozygous triploid LLR male inherited only two allele combinations. There are two possible explanations for this pattern: first, the amplified loci may be too close to each other on the genome to make recombination between them likely or, second, there was truly no recombination between the two L-genomes in this individual. In contrast, in the double heterozygous LRR female four allele combinations were inherited by the offspring in more or less equal proportions. This is strong evidence that, in this case, recombination between the loci has taken place. But in order to get an improved picture of what is going on in these triploid animals and whether recombination is happening in all triploid individuals or only in some, more crossings with double heterozygous animals have to be performed. The need for more crossings also arises from the fact that the type of gamete production observed in our experiment is not universal. Several exceptions have been described previously, such as the production of diploid sperm (Uzzell et al. 1977, Tunner 2000) and the formation of diploid eggs by triploid females (Günther et al. 1979).

The mean fertilization rate and subsequent early development variables show that only about one third of the offspring survives the first larval phase and develops normally. Since rearing conditions were chosen to be benign, this rate seems quite low compared to other amphibian species (Beattie et al. 1991, Sagvik et al. 2005). However, such high offspring mortality is not unique to the Swedish *R. esculenta* populations, but has been shown for the Palearctic water frog system in several other studies (Berger 1967, Eikhorst 1987, Christiansen et al. 2005). From another study on these Swedish populations (chapter 4) we know that these populations are genetically depleted, probably due to their location at the edge of the species' distribution. We therefore tested whether outcrossing with Polish frogs has any effect on offspring viability. We expected a positive effect of outcrossing if accumulated mutations within the Swedish genome cause early developmental problems or death, but a negative effect if the Polish genomes are genetically so different from the Swedish ones that genetic incompatibilities arise (Sagvik et al. 2005). Our results showed clearly that the Polish females outcrossed with Swedish males had low

fertilization success, low hatchling survival and a low proportion of normally developing tadpoles (Fig. 6). In contrast, outcrossing Swedish females with male frogs from Poland did not have significant effects on any developmental trait. Many eggs stripped from the Polish females looked small and somehow unripe. It has been shown that not all females spawn every year (Reyer et al. 2004), and if females are stressed before crossing/mating they might react by postponing reproduction and not maturate the eggs completely. We conclude that the low fertilization success of the Polish females is due to a stress factor imposed by long transportation, rather than by genetic incompatibility. The similar values for early development variables in pure Swedish crosses and in crosses between Swedish mothers and Polish fathers indicate that in our study there was no direct inbreeding or outbreeding effects on early development of tadpoles.

Our findings show that the hybrid genotypes (LR, LLR and LRR) do not differ significantly in their ability to produce fertilized eggs and normally developed tadpoles. Therefore, in a pure hybrid population with random mating genotypes should contribute equally to the next generation. There were, however, individual differences. Some individuals failed almost completely to reproduce, independent of their genotype. Considering the complex processes during gametogenesis in hybridogenetic frogs (Günther 1975), this is not astonishing. It has also been shown in other water frog mating systems that some individuals fail to produce viable gametes (Uzzell et al. 1977, Eikhorst 1987).

For later larval stages we assume that survival is not so much determined by the genotypes of the parents, but more by the genotype of the offspring itself. Our data indicate that, once a tadpole has hatched and looks normal, its chance of metamorphosing is high and there is no direct selection against parental genotypes (LL and RR), at least not under benign conditions. However, these two parental genotypes developed slower which translated into a longer time until metamorphosis and lower weight at metamorphosis (Figs. 7 and 8). Other studies on amphibians have shown that these life-history traits have an influence on the fitness at later life stages by either directly reducing survival or reducing reproductive success (Semlitsch et al. 1988, Morey and Reznick 2001, Altwegg and Reyer 2003). Since larval period tends to be shorter and winters longer further north such delayed life-history traits might even have a stronger influence on later survival. In contrast to high survival at late larval stages, the early life phases of the froglets seemed to be much harsher. Less than one third of the individuals that started to metamorphose survived until after the first hibernation (Fig. 9a). The RR genotype seemed to survive worst. Due to low sample size at this stage we were not able to establish completely

the sensitive post-metamorphic life-stages for each of the different genotypes. However, the two triploid genotypes differed in their post-metamorphic mortality as LRR died mainly before hibernation and LLR mostly during hibernation (Fig. 9b). Hibernation conditions were rather artificial; it is therefore difficult to make implications about selective mortality acting on these genotypes in nature. Nevertheless, our experiment showed that the two parental genotypes (LL and RR) which are not found among the adults in Southern Sweden were formed in artificial crossings and not explicitly selected against under experimental conditions.

In order to obtain sufficient number of male and female genotypes needed for the crossings, parents were collected from three different source ponds. As a result, our offspring sample contained larvae with parents from different ponds and those with parents from the same pond. This mix could potentially explain the fairly high survival of offspring with parental genotypes. However, a closer look at the crosses revealed that all LL offspring were from parents coming from neighboring ponds and all RR offspring even originated from within-pond crosses. Thus, it seems realistic to assume that the adult frogs parenting these crosses actually mate in nature. We therefore predict that these genotypes are also formed in these natural ponds, but do not survive because pond conditions being harsher than our benign experimental conditions.

Acknowledgements

We wish to thank H.-U. Reyer, D. Christiansen, T. Garner, J. Loman, H. Hotz and G. Guex for help with the experiment. Thanks to J. Plötner who provide the Polish frogs for the experiment. Many thanks also to S. Röthlisberger for her valuable help in the lab. B. Baldetorp, M. Johansson and E. Niederer kindly provided advice for flow cytometry analysis. We also wish thank to J. Van Buskirk for statistical advice and H.-U. Reyer and C. Vorburger who gave helpful comments on earlier versions of the manuscript. This work was supported by Swiss NF grant No. 31-64004.00 to H.-U. Reyer.

Author contributions

M.A. and C.J. contributed equally to this work. Both authors carried out all field- and lab work together. M.A. performed statistical analyses and wrote the paper. Both authors discussed the results and C.J. commented on the manuscript.

References

Abt, G. 2003. Pond use, patterns of reproduction and juvenile recruitment in a mixed waterfrog population. PhD-Thesis. University of Zurich, Switzerland.

Alford, R. A., and R. N. Harris. 1988. Effects of larval growth history on anuran metamorphosis. The American Naturalist **131**:91-106.

Alford, R. A., and G. D. Jackson. 1993. Do cephalopods and larvae of other taxa grow asymptotically? The American Naturalist **141**:717-728.

Altwegg, R., and H.-U. Reyer. 2003. Patterns of natural selection on size at metamorphosis in water frogs. Evolution **57**:872-882.

Alves, M. J., M. M. Coelho, and M. J. Collares-Pereira. 2001. Evolution in action through hybridisation and polyploidy in an Iberian freshwater fish: a genetic review. Genetica **111**:375-385.

Arnold, M. L., and S. A. Hodges. 1995. Are natural hybrids fit or unfit relative to their parents? Trends in Ecology and Evolution **10**:67-71.

Barton, N. H. 2001. The role of hybridization in evolution. Molecular Ecology **10**:551-568.

Barton, N. H., and G. M. Hewitt. 1985. Analysis of hybrid zones. Annual Review of Ecology and Systematics **16**:113-148.

Beattie, R. C., R. J. Aston, and A. G. P. Milner. 1991. A field study of fertilization and embryonic development in the common frog (*Rana temporaria*) with particular reference to acidity and temperature. Journal of Applied Ecology **28**:346-357.

Berger, L. 1967. Embrional and larval development of F_1 generation of green frogs different combinations. Acta Zoologica Cracoviensia **12**:123-160.

Berger, L., and H. Roguski. 1978. Ploidy of progeny from different egg size classes of *Rana esculenta* L. Folia Biologica Krakow **26**:231-248.

Berger, L., M. Rybacki, and H. Hotz. 1994. Artificial fertilization of water frogs. Amphibia-Reptilia **15**:408-413.

Christiansen, D., K. Fog, B. V. Pedersen, and J. J. Boomsma. 2005. Reproduction and hybrid load in all-hybrid populations of *Rana esculenta* water frogs in Denmark. Evolution **59**:1348-1361.

Eikhorst, R. 1987. Der Laich des Teichfrosches *Rana esculenta* Linnaeus, 1758 in einer reinen Bastardpopulation (Anura: Ranidae). Salamandra **23**:122-131.

Fog, K., A. Schmedes, and D. Rosenørn de Lasson. 1997. Nordens padder og krybdyr. G.E.C. Gads forlag, Copenhagen.

Gosner, K. L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica **16**:183-190.

Graf, J.-D., and M. Polls Pelaz. 1989. Evolutionary genetics of the *Rana esculenta* complex. Pages 289-302 *in* R. M. Dawley and J. P. Bogart, editors. Evolution and Ecology of Unisexual Vertebrates. New York State Museum, Albany, New York, USA.

Guex, G. D., H. Hotz, T. Uzzell, R. D. Semlitsch, P. Beerli, and R. Pascolini. 2001. Developmental disturbances in *Rana esculenta* tadpoles and metamorphs. Mitteilungen aus dem Museum für Naturkunde Berlin, Zoologische Reihe **77**:79-86.

Günther, R. 1975. Untersuchungen der Meiose bei Männchen von *Rana ridibunda* Pall., *Rana lessonae* Cam. und der Bastardform "*Rana esculenta*" L. Biologische Zentralbibliothek **94**:277-294.

Günther, R. 1990. Die Wasserfrösche Europas. A. Ziemsen Verlag, Wittenberg.

Günther, R., T. Uzzell, and L. Berger. 1979. Inheritance patterns in triploid *Rana "esculenta"* (Amphibia, Salientia). Mitteilungen aus dem Zoologischen Museum Berlin **55**:35-57.

Hedges, S. B., J. P. Bogart, and L. R. Maxson. 1992. Ancestry of unisexual salamanders. Nature **356**:708-710.

Jakob, C. 2007. Structure and dynamics of pure hybridogenetic water frog populations of *Rana esculenta* in Southern Sweden. PhD-Thesis. University of Zurich, Switzerland.

Licht, P., D. Porter, and R. P. Millar. 1987. Specificity of amphibian and reptilian pituitaries for various forms of gonadotropin-releasing hormones in vitro. General and comparative endocrinology **66**:248-255.

Mantovani, B., and V. Scali. 1992. Hybridogenesis and androgenesis in the stick-insect *Bacillus rossius-grandii benazzii* (Insecta Phasmatodea). Evolution **46**:783-796.

Mayr, E. 1942. Systematics and the origin of species. Columbia University Press, New York.

Mc Creery, B. R., and P. Licht. 1983. Induced ovulation and changes in pituitary responsiveness to continuous infusion of gonadotropin-releasing hormone during the ovarian cycle in the bullfrog, *Rana catesbeiana*. Biology of Reproduction **29**:863-871.

Morey, S., and D. Reznick. 2001. Effects of larval density on postmetamorphic spadefoot toads (*Spea hammondii*). Ecology **82**:510-522.

Ogielska, M. 1994. *Rana esculenta* developmental syndrome: Fates of abnormal embryos from the first cleavage until spontaneous death. Zoologica Poloniae **39**:447-459.

Quattro, J. M., J. C. Avise, and R. C. Vrijenhoek. 1992. Mode of origin and sources of genotypic diversity in triploid gynogenetic fish clones (*Poeciliopsis:* Poeciliidae). Genetics **130**:621-628.

Reyer, H.-U., B. Niederer, and A. Hettyey. 2003. Variation in fertilisation abilities between hemiclonal hybrid and sexual parental males of sympatric water frogs (*Rana lessonae*, *R. esculenta*, *R. ridibunda*). Behavioral Ecology and Sociobiology **54**:274–284.

Reyer, H.-U., M.-O. Wälti, I. Bättig, R. Altwegg, and B. Hellriegel. 2004. Low proportions of reproducing hemiclonal females increase the stability of a sexual parasite-host system (*Rana esculenta*, *R. lessonae*). Journal of Animal Ecology **73**:1089-1101.

Sagvik, J., T. Uller, and M. Olsson. 2005. Outbreeding depression in the common frog, *Rana temporaria*. Conservation Genetics **6**:205-211.

SAS Institute. 2002-2003. Version 9.1.3 SP3 for Windows. SAS Institute Inc., Cary, NC.

Schultz, R. J. 1969. Hybridization, unisexuality, and polyploidy in the teleost *Poeciliopsis* (Poeciliidae) and other vertebrates. The American Naturalist **103**:605-619.

Semlitsch, R. D., and H.-U. Reyer. 1992. Performance of tadpoles from the hybridogenetic *Rana esculenta* complex: Interactions with pond drying and interspecific competition. Evolution **46**:665-676.

Semlitsch, R. D., D. E. Scott, and J. H. K. Pechmann. 1988. Time and size at metamorphosis related to adult fitness in *Ambystoma talpoideum*. Ecology **69**:184-192.

Stahel, W. A. 1995. Statistische Datenanalyse: Eine Einführung für Naturwissenschaftler, 4th edition. Vieweg & Sohn Verlagsgesellschaft mbH, Braunschweig.

Thurnheer, S. 1999. Direct and behaviorally mediated effects of predators on water frog tadpoles. PhD-Thesis. University of Zurich, Switzerland.

Tunner, H. G. 1974. Die klonale Struktur einer Wasserfroschpopulation. Zeitschrift für zoologische Systematik und Evolutionsforschung **12**:309-314.

Tunner, H. G. 2000. Evidence for genomic imprinting in unisexual triploid hybrid frogs. Amphibia-Reptilia **21**:135-141.

Uzzell, T., R. Günther, and L. Berger. 1977. *Rana ridibunda* and *Rana esculenta*: a leaky hybridogenetic system (Amphibia Salientia). Proceedings of the Academy of Natural Sciences of Philadelphia **128**:147-171.

Vinogradov, A. E., L. J. Borkin, R. Guenther, and J. M. Rosanov. 1990. Genome elimination in diploid and triploid *Rana esculenta* males: cytological evidence from DNA flow cytometry. Genome **33**:619-627.

Vorburger, C. 2001. Fixation of deleterious mutations in clonal lineages: evidence from hybridogenetic frogs. Evolution **55**:2319-2332.

Vrijenhoek, R. C. 1994. Unisexual fish: Model systems for studying ecology and evolution. Annual Review of Ecology and Systematics **25**:71-96.

Table 1. One-way ANOVA testing for differences in early developmental variables between crosses with two Swedish parents (Swe/Swe), crosses of Swedish females with Polish males (Swe/Pol) and crosses of Polish females with Swedish males (Pol/Swe).

		Fertilization success		Hatchling survival		Normal tadpoles	
Source of variation	df	*F*	*P*	*F*	*P*	*F*	*P*
Cross type	2	13.75	**< 0.001**	1.87	0.165	4.87	**0.012**

Table 2. One-way ANOVA testing for the effect of offspring genotype on larval survival to metamorphosis (MM), larval growth rate, mass at metamorphosis, length of larval period until metamorphosis and on post-larval survival rate until after first hibernation.

		Larval survival rate		Larval growth rate		Mass at MM		Larval period until MM		Post-larval survival rate	
Source of variation	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Offspring genotype	4	2.98	0.080	2.50	0.116	4.16	**0.027**	4.79	**0.024**	0.50	0.736

Table 3. Mixed-model nested analyses of variance (ANOVAs) for fertilization success, hatchling survival and proportion of normal tadpoles in relation to female and male genotypes, their interaction and individual females and males nested within their genotype. Proportions were arcsine-square root transformed before analyses.

		Fertilization success		Hatchling survival		Normal tadpoles	
Source of variation	df	*F*	*P*	*F*	*P*	*F*	*P*
Female genotype	2	0.79	0.493	0.41	0.672	1.25	0.338
Male genotype	1	0.00	0.961	0.13	0.733	0.01	0.926
Female genotype * male genotype	2	17.73	**0.002**	3.54	0.086	4.20	0.063
Female (genotype)	6	49.10	**< 0.001**	2.99	0.089	6.15	**0.015**
Male (genotype)	5	45.37	**< 0.001**	15.17	**0.001**	12.54	**0.002**

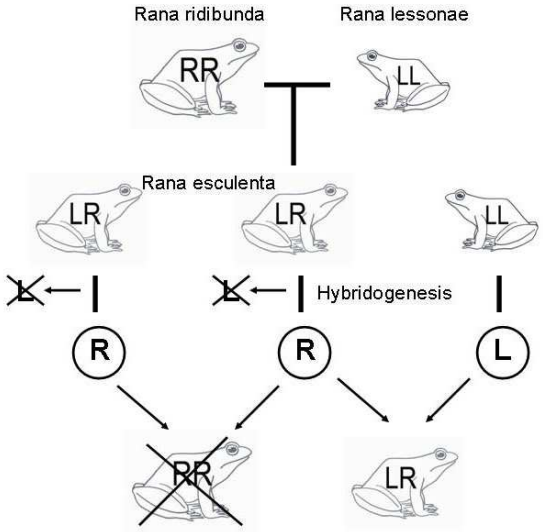

Figure 1. Mating pattern and resulting offspring in a typical *lessonae-esculenta* system. The hybrid *Rana esculenta* (LR) is formed by matings between *Rana ridibunda* (RR) and *Rana lessonae* (LL). The hybrid eliminates one of the genomes (L) from the germline and passes the other (R) clonally to the offspring ("hybridogenesis"). By mating with a *R. lessonae* the hybrid regains the missing genome and produces another hybrid. Offspring from pure hybrid matings usually do not survive in the LE-system.

Males / Females	LR (R)	LLR (L)	LRR (R)
LR (LR) (R)	LRR / RR	LLR / LR	LRR / RR
LLR (L)	LR	LL	LR
LRR (R)	RR	LR	RR

Figure 2. Schematic figure showing the production of gametes for females and males of the three genotypes found in pure hybrid systems of *R. esculenta*. Offspring with the parental genotypes (LL and RR) are in grey boxes, because they are not found among reproducing adults.

		Sweden		Poland	
	Males (n =)	LLR(6)	LR (4)	LL (4)	LR (4)
	Females (n =)				
Sweden	LLR (4)	X	X	O	O
	LR (4)	X	X	O	O
	LRR (4)	X	X	O	O
Poland	LL (1)	O	O		
	LR (3)	O	O		

Figure 3. Crossing design including Swedish and Polish individuals in order to test for possible outcrossing/inbreeding effects. X represent crosses with both Swedish parents, O represent crosses where one parent was Polish.

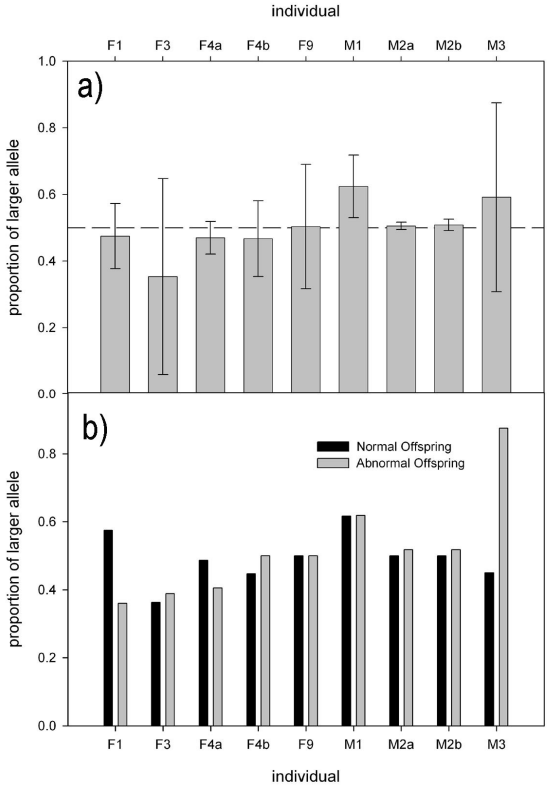

Figure 4. a) Relative inheritance frequencies of the larger of two alleles in 7 different triploid hybrid individuals (F: Females, M: Males). Error bars indicate 95% confidence intervals; the dashed line shows 0.5 proportion expected under random allele segregation. b) Relative inheritance frequencies of the larger allele in normal and abnormal offspring.

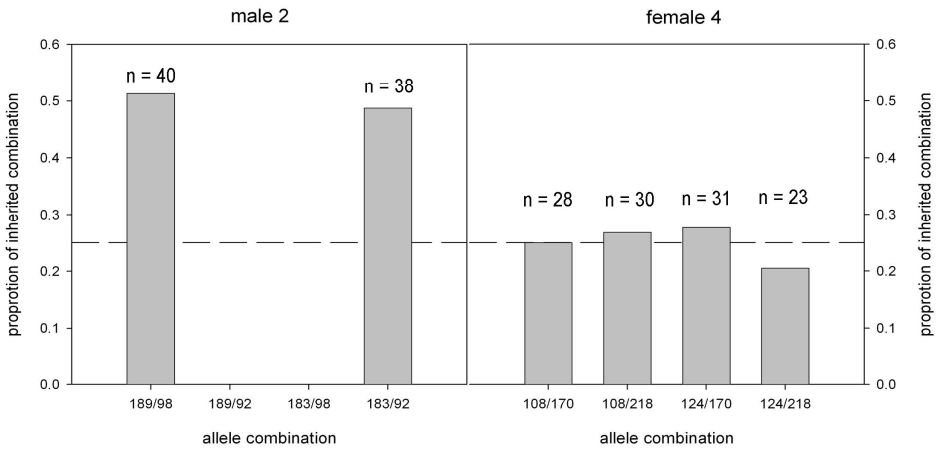

Figure 5. Relative inheritance frequencies of allele combinations in offspring of double heterozygous triploids. Male 2 is an LLR individual with alleles 92/98 in locus RE1CAGA10 and alleles 183/189 in locus Ca18. Female 4 has the genotype LRR with alleles 108/124 in locus Re1CAGA10 and alleles 170/218 in locus Re2CAGA3. The dashed line indicates a proportion of 0.25 expected under random segregation.

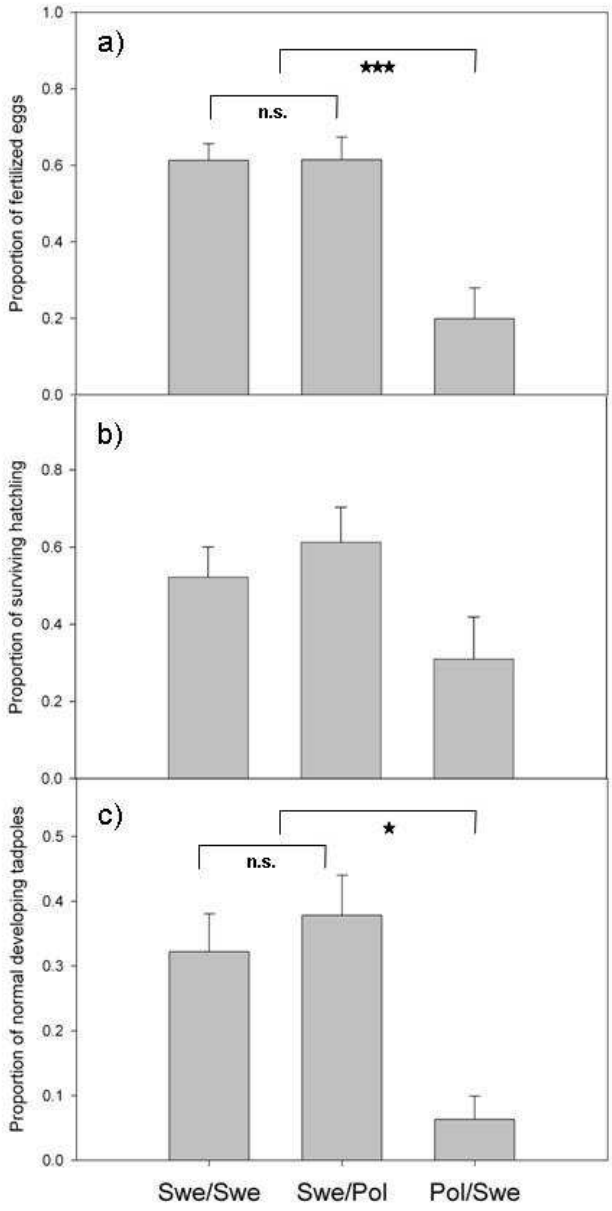

Figure 6. Differences in a) fertilization success, b) hatchling survival and c) proportion of normal developing tadpoles between crosses with both parents from Sweden (Swe/Swe, n = 27), crosses with Swedish females and Polish males (Swe/Pol, n = 17) and crosses with Polish females and Swedish males (Pol/Swe, n = 10). Bars represent means with standard errors. ★★★ indicate $P < 0.001$ and ★ $P < 0.05$.

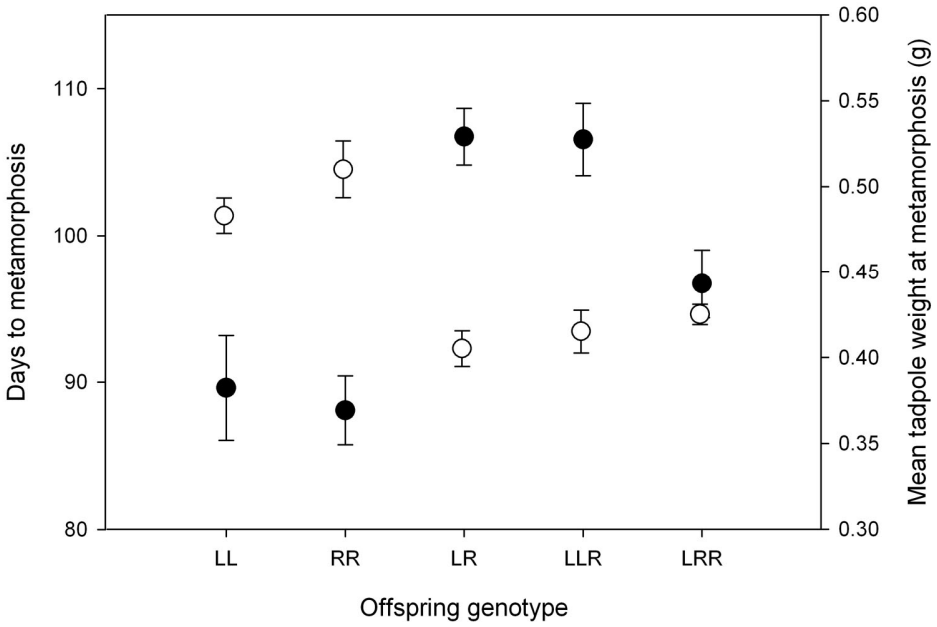

Figure 7. Mean tadpole mass (●) at metamorphosis (stage 42) and mean length of larval period until metamorphosis (○) for the five different offspring genotypes. Error bars represent ± 1 SE.

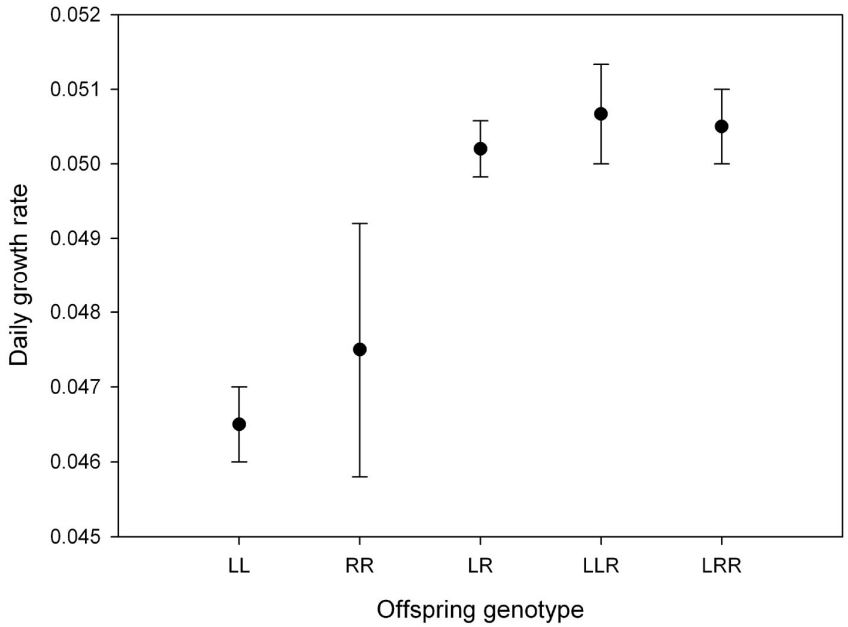

Figure 8. Mean daily growth rate ● as a function of offspring genotype. Error bars represent ± 1 SE.

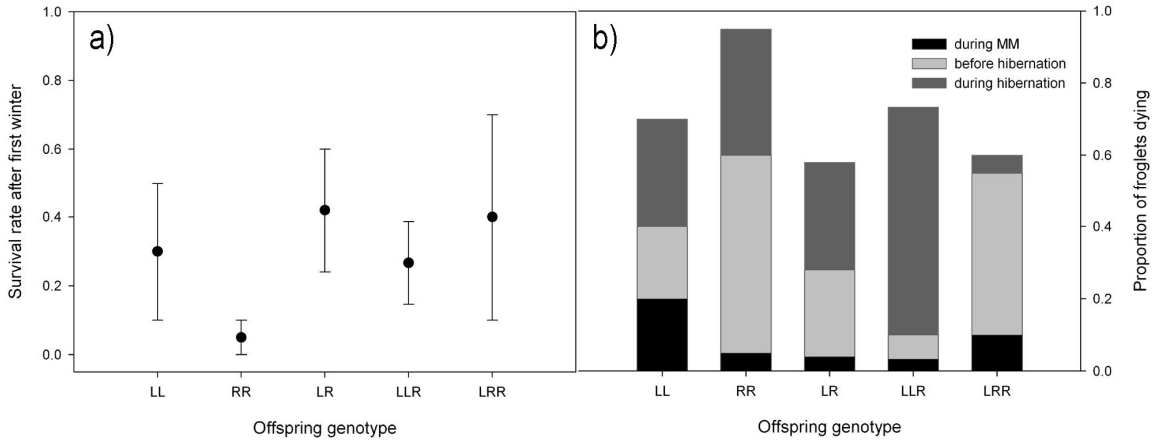

Figure 9. a) Mean survival rate (± 1 SE) until after the first hibernation. b) Proportions of froglets that died during the three time periods indicated in the upper right corner.

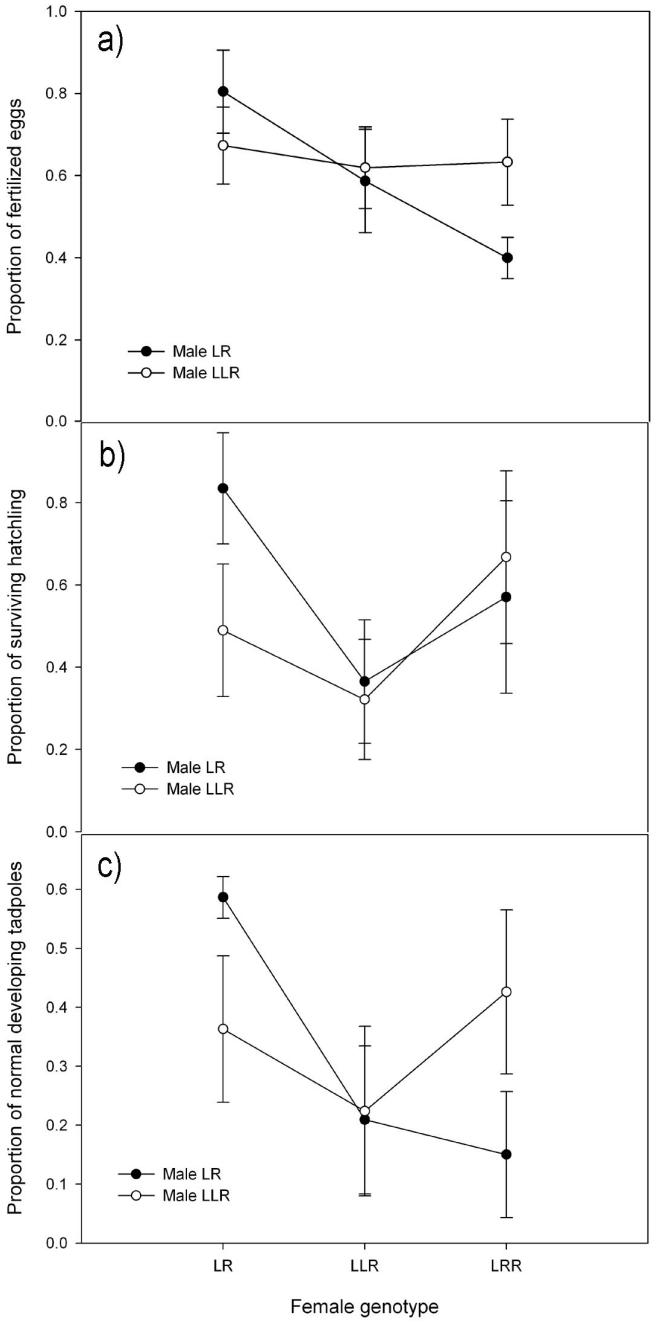

Figure 10. Results from the interaction of female and male genotype on a) fertilization success b) hatchling survival and c) proportion of normal developing tadpoles. Values represent means ± 1 SE.

CHAPTER 2

Genotypic composition of *Rana esculenta* population systems in Sweden

CHRISTIAN JAKOB, MARTINA ARIOLI & HEINZ-ULRICH REYER

***Abstract*.**-Pure hybrid populations of the water frog *Rana esculenta* are exceptional in many aspects. Besides overcoming the alleged hybrid disadvantages, *R. esculenta* (LR genotype) normally lives in obligate sympatry with one of its parental species, either *R. ridibunda* (RR) or *R. lessonae* (LL), parasitizing it sexually due to its special reproductive mode of hybridogenesis. In pure hybrid populations, triploid hybrid specimens provide the system's stability and viability instead of the parental species. We could show that in Southern Sweden, contrary to assumptions made by previous investigators, the three main genotypes (diploid LR and the two triploid forms LLR and LRR) occur together simultaneously in most of the ponds. Low frequencies of tetraploid and mosaic animals were also unexpectedly present in some ponds, as well as other special genotypes. Genotypic compositions changed significantly in the years 2002-2004. The amount of LR genotypes was steadily increasing, while mainly LLR proportions were decreasing. With the exception of 4 animals, all adult frogs were of a non-parental genotype. Finally, we could show a strongly skewed sex ratio for LRR and RR animals towards females and less pronounced for LLR and LLRR genotypes towards males, providing some support for the theory of L-genome-linked male determining factors.

In the Östergötland district, a newly discovered population could be identified as mixed population of *R. lessonae* and diploid *R. esculenta*. This is the first report of this population type in Sweden.

Introduction

The European edible frog, *Rana* kl *esculenta* L., is regarded as one of the key examples of how taxa of hybrid origin can defy the often proposed “evolutionary dead-end”: Stemming from hybridizations between the pool frog *R. lessonae* Camerano (genotype LL) and the lake frog *R. ridibunda* Pallas (RR), *R. esculenta* (LR) is actually the most widespread water frog taxon in Europe and was shown to form apparently long-term stable populations, mostly in sympatry with one of its parental species (Uzzell 1982). The reason for the common coexistence of *R. esculenta* with either *R. lessonae* or *R. ridibunda* lies in its reproductive mode. The edible frog reproduces hybridogenetically (also known as hemiclonally, see Schultz 1969), a reproductive mode also known from certain fishes, stick insects, and salamanders (Vrijenhoek 1989, Hedges et al. 1992, Mantovani and Scali 1992). By excluding one half of its genome prior to meiosis, *R. esculenta* transmits the other part clonally to its gametes (reviewed in Graf and Polls Pelaz 1989). In the case of mixed populations of *R. lessonae* and *R. esculenta* (a so-called LE-system), the edible frog excludes the L-part of its genome and passes on the R-part clonally. In *ridibunda-esculenta* systems (RE-systems), gamete exclusion works conversely (reviewed in Plötner 2005). Homotypic mating between *R. esculenta* lead to inviable offspring of parental genotype (LL or RR, respectively) because through repeated clonal inheritance, deleterious mutations have accumulated which are then present in a homozygous state (a principle called Mullers ratchet, Muller 1964). In general, *R. esculenta* is therefore obligatory sympatric with one of its parental species, which it then sexually parasitizes.

Besides the common LE- and RE- systems, several exceptions are known, for example pure hybrid systems. In pure hybrid systems, the parental genotypes (LL and RR) are absent among the adults. Instead, triploid LLR and LRR animals, occurring together with diploid LR, take over the role of the parental genotypes by excluding the genome in least copy number and propagating the double-copy genome after normal meiosis (Günther et al. 1979). Such pure hybrid populations are mainly known along the northern distribution range of *R. esculenta* (Günther 1990, Plötner 2005), e.g., in Northern Germany (Günther and Plötner 1990, Berger and Berger 1994), Poland (Ogielska et al. 2001, Rybacki and Berger 2001), Denmark (Fog 1994, Christiansen et al. 2005), and Sweden (Ebendal 1979).

In Sweden, the focus area of this study, the following water frog population systems have been reported previously:

a. Pure populations of the pool frog, *R. lessonae*, along the Northern Uppland coast, Central Sweden (area 1 in Fig. 1).
b. Pure hybrid populations of the edible frog, *R. esculenta*, in South Western Skåne (Scania), Southern Sweden, some 600 km south of the *R. lessonae* populations (area 2 in Fig. 1).

Additionally, several water frog localities lying between the aforementioned population systems were reported earlier along the Eastern Swedish coast (areas 3-7 in Ebendal 1979).

The isolated occurrence of *R. lessonae* in Central Sweden has sparked the interest of many scientists, resulting in a multitude of publications including investigations of their relationship with other *R. lessonae* populations in Northern Europe (Ebendal and Uzzell 1982, Sjögren Gulve 1991, 1994, Wycherley et al. 2001, Zeisset and Beebee 2001, Tegelström and Sjögren-Gulve 2004, Snell et al. 2005). Therefore, their genotypic composition is well known and undisputed. In this publication we concentrate on the other localities. After some preliminary investigations in Southern Sweden by (Ebendal and Uzzell 1982), no further systematic studies of these pure hybrid populations were conducted. Sample sizes were generally small and traditional methods used for determination of genotypes (morphology, morphometry, serum electrophoresis) often lack discriminatory powers (Jakob 2007). Finally, the actual composition of the additional water frog communities in Eastern Skåne and along the Eastern Swedish coast mentioned in Gislén and Kauri (1959) and Ebendal (1979) remains unknown because they have apparently gone extinct, either already before 1979, or until the late 1990s (Kvindall 1998, J. Pröjts, pers. comm.). In the course of an inventory of two presumed *R. esculenta* localities, Jan Pröjts of Ekologgruppen i Landskrona AB could confirm acoustically the presence of water frogs in Lake Vindommen at Hannäs (Östergötland district), which was detected by locals in 1975 (Söderbäck 1984). In 2004, we discovered another population of water frogs in a pond near Hannäs.

The distribution maps in Günther (1990) and in Plötner (2005) show therefore an inaccurate picture for Swedish water frogs today, because they rely on historical data, whereas Fog et al. (1997) use for their distribution map of *R. esculenta* only the confirmed localities mentioned in Ebendal (1979), without the East Skåne populations.

This study is the first large-scale investigation on the composition of pure hybrid populations of the *R. esculenta* water frog complex (EE), and also reports the first finding of a third water frog population system in Sweden, the LE-system.

Methods

Skåne samples

The sampling was conducted in the years 2002-2004 in an area located in South-Western Skåne (Scania), Southern Sweden (area 2 in Fig. 1).

Prior to and during the first year, we have investigated a total of approximately 140 ponds in the region, finally selecting 23 of them for detailed analysis. Selection was based on criteria such as accessibility of the pond, number of frogs present, as well as the practicability of catching frogs in the pond (depth, riparian morphology) and the possibility to allow a representative sampling of the population (pond size). Within the 23 ponds, a subsample of 12 ponds ("core ponds") was also surveyed in 2003 and 2004. The ponds were sampled at least twice in the season (May to July) at variable time intervals. Including a total of 514 recaptures, we caught 973 frogs in 2002, (mean n per pond: 42 ± 12), 1180 in 2003 (98 ± 33) and 1080 in 2004 (90 ± 18). In 2003, one newly dug pond was sampled once in the season in addition to the 12 core ponds (n=37). In 2004, 9 additional locations along the edge of the *R. esculenta* distribution in Southern Sweden were sampled once in June or July (283 frogs, mean n per pond: 31 ± 3). For a list of the sample sites, see table 1.

In 2002, only seemingly sexually mature frogs were collected, i.e., frogs larger than about 45mm (snout-vent length), whereas in the following years, also subadult frogs entered the sample.

Östergötland samples

In June 2004, the pond Lindalsgöl near Hannäs in Östergötland (area 3 in Fig. 1) was sampled once (40 frogs).

Sampling procedures

Frogs were caught by hand during night time with the help of a flashlight and transported to the Stensoffa field station of the University of Lund. Within 24 hours, the frogs were measured, weighed, and individually marked with a RFID PIT tag (Trovan ID101, Trovan Ltd., UK), except for animals from populations that were sampled only once and subadults smaller than 30mm. One phalanx of the fourth toe was clipped for DNA analysis and stored in Ethanol at -20℃ until analysis. Additionally, about 30-50µl blood was taken from a web vein for flow cytometric analysis with a heparinized capillary tube (70µl Micro-Hematocrit Capillary Tubes, VWR International, West Chester USA) and stored in citrate buffer (D-(+)-glucose 475 mM, Sigma G8270; trisodium citrate 40 mM, Sigma-Aldrich S4641; dimethyl

sulfoxide 5%, Sigma D8418; pH 7.6) at -80°C until a nalysis. Within 24 hours, frogs were released at their capture sites.

Genotype determination

Genotype determination followed the procedures described in Jakob (2007) by means of flow cytometry of nucleated red blood cells, and by gene dosage effects in the microsatellite primers Ca1b5 (Garner et al. 2000), Ca1b6, Ga1a19 (chapter 4) and Res16 (Zeisset et al. 2000). Samples yielding contradictory results were re-analyzed and, if contradictory results were persistent, referred to as “Mixed2n” for diploid and “Mixed3n” for triploid animals. Samples that could not be assigned to a genotype because of insufficient sample quality (tissue and/or blood) were left out of the analysis. Specimen with single missing microsatellite primer alleles were assigned to a genotype according to the other microsatellite primers and flow cytometric results, but were recorded specifically. Mosaic animals (with differing DNA contents in different cells) were detectable by flow cytometry only, showing 2 distinct peaks of luminescence (see Jakob 2007). By the same method it is also possible to determine aneuploidy with an incomplete set of chromosomes.

Statistical analyses

To test for significant changes in global genotype composition between the years, we have performed ANOVAs pooled over all samples from all ponds, grouped by males, females, adults and juveniles for the three main genotypes LR, LLR and LRR. To test for pond and year effects on genotype frequency, we performed an ANOVA on the sub-sample of the 12 core ponds (which were sampled over all three years). To test for possible systematic temporal shifts in genotype composition within a year (e.g., some genotypes appearing and leaving earlier in the breeding season compared to others), an ANOVA with relative genotype frequency classified by sampling month and controlled for year effects was performed for males, females, juveniles and adults pooled over the core ponds.

Statistical analyses were performed with SAS 9.1.3 SP3 for Windows (© 2002-2003 SAS Institute Inc., Cary, NC, USA.), percentage data was arcsine squareroot-transformed before statistical analysis ($x' = \arcsin\sqrt{x}$) to assure a nearly-normal distribution. Graphs were produced using SigmaPlot 2002 v8.02 for Windows (© 1986-2001 SPSS Inc., Chicago, IL, USA).

Results

The main adult genotypes found in Southern Sweden were diploid LR, together with triploid LLR and LRR. Occasionally, also tetraploid adults were found, as well as specimen classified as “mixed” because results from genotype determination methods were consistently contradictory. With the exception of pond 089 in 2003 and pond 154 in 2004, where four individuals of the parental RR genotype were found, parental genotypes (LL or RR) were completely absent from adult samples, although they were occasionally found among juveniles. When looking at combined samples in 2002, 22 out of 23 sampled ponds had an excess in triploid animals (LLR and LRR genotypes combined, Fig. 2). The amount of diploid adult LR animals varied between 7.7% in pond 001 and 53.1% in pond 032 (mean: 28.9%, stdev: 11.6%). Two ponds (123 and 137) consisted only of LR and LLR animals, in a further pond (138), only LR and LRR animals were found. In all other ponds, all three main genotypes were present at the same time at varying rates. The fact that the three main genotypes were present in the majority of the sampled ponds at the same time was true also for 2003, (except for pond 001: no LRR, see Fig. 3), and for 2004 (except for pond 154: no LLR; and pond 161: no LRR; see Fig. 4). Relative genotype frequencies within a pond can change quite dramatically within and between years (as can be seen in Figs. 5, 6, 7, and 8, shown for our 12 core ponds). Also, overall genotype frequencies changed over the years (see Fig. 9): When performing an ANOVA (PROC GLM in SAS) over all pooled samples considering only LR, LLR and LRR genotypes, there was a significant year-effect on the percentage of caught LR genotypes (and consequently, the relative amount of diploid animals in the samples) between the years (2002-2004) for adult frogs ($P < 0.0001$, $F = 11.60$, df = 2), female frogs ($P < 0.01$, $F = 8.41$, df = 2), male frogs ($P < 0.05$, $F = 4.34$, df = 2), and juvenile frogs (only 2003 and 2004 samples; $P < 0.05$, $F = 6.95$, df = 1). Post-hoc Scheffé’s tests showed that significant changes in relative LR, respectively diploid frequencies occurred between 2002 and 2004, and between 2003 and 2004. The relative increase of LR animals was mostly at the cost of LLR genotypes. When performing the same analysis for them, there were significant differences for adults ($P < 0.0001$, $F = 13.68$, df = 2), females ($P < 0.05$, $F = 8.85$, df = 2), males ($P < 0.0001$, $F =13.43$, df = 2), but not for juveniles ($P > 0.05$, $F = 0.47$, df = 1). LRR frequencies in the pooled sample did not significantly change over the years, however (data not shown), except for juvenile frogs ($P < 0.05$, $F = 4.32$, df = 1).

While adult LR and mixed genotypes consisted about equally of males and females, the sex ratios of the other genotypes were skewed (shown in Fig. 10). LRR,

RR and Mosaic animals exhibited a strong female bias, whereas LLR and LLRR animals were, to a lesser extent, biased towards males.

When comparing the 12 core ponds which were sampled every year by means of an ANOVA (PROC GLM in SAS), performed for each genotype, significant effects of pond, year, and also interaction effects between both were found for some groups (Table 2). There were genotype frequency changes within each year between sampling events. But when testing each pond for year and sampling month effects, there was no systematic effect of sampling month on genotype frequency, except for pond 014, where a significantly higher frequency of LR adult frogs was caught in May than in June ($P < 0.05$, $F = 23.40$, df = 1), and for LR females in pond 032 which were caught with lowest frequency in June, highest in July and medium frequency in May ($P < 0.05$, $F = 3.68$, df = 2). When testing over all ponds, genotype frequencies were not affected by sampling month.

In contrast to the pure hybrid, diploid-polyploid population system in Skåne, the population in Östergötland is an LE-system (figure 11), consisting of only diploid *R. esculenta* (LR), together with diploid *R. lessonae* (LL). This population system is found also in large parts of Central Europe.

Discussion

Skåne

We could show that *R. esculenta* forms pure hybrid populations in Skåne, as was suggested in Ebendal (1979) and Ebendal and Uzzell (1982). Because of the low sample sizes analyzed in these publications, the authors had to include the possibility for the presence of adult genotypes in low frequency. With our analysis of nearly 3000 individual specimen, we can rule this possibility out. Parental genotypes are formed by non-assortative mating, but their frequency in the different life stages is gradually decreasing (chapter 3), until they are no longer present in the adult samples. There were 4 exceptions in our data set: 1 female from pond 089 and 3 females from pond 154, which all exhibited RR genotypes. While the animal from 089 was most probably a juvenile animal that was misclassified by our arbitrary size limit, the animals from 154 were definitely adults. The concentrated presence of these RR genotypes in this pond (situated on a golf course) is puzzling and should be monitored. Although introduction of RR animals cannot be ruled out completely, it is nevertheless improbable judging from microsatellite and mtDNA data (chapter 5).

In the past, the (now extinct) populations in South Eastern Skåne have been described either as “morphologically slightly similar” to RR (Ebendal 1979), or have been found to be of the LR and LRR genotype (Ebendal and Uzzell 1982). The specimens from South Western Skåne, however, have always been described as LR and LLR genotypes. We could show that in fact all three main genotypes (LR, LLR, and LRR) occur in most of the ponds simultaneously in varying proportions. Contrary to the simple and reportedly most widespread situation of LR-LLR populations modelled by Som and Reyer (2006), LRR animals are quite common and, as shown by Jakob 2007 and chapter 3, are viable. That the three main genotypes may occur together in the same pond was also shown by Christiansen et al. (2005) for some pure hybrid populations in Denmark, although one triploid genotype was always dominant over the other. In contrast to this publication, however, we have found also adult tetraploid animals in our sample, thanks to the incorporation of flow cytometric analyses for ploidy determination in our study. Tetraploid animals may provide a step towards the formation of a new, independent species by reintroducing normal meiosis. In the diploid/polyploid hybrid *Squalius alburnoides* system, tetraploid animals are regarded as a possibility for a return to normal sexual reproduction, although they are in low frequency (Pala and Coelho 2005). Although Vrijenhoek (2006) has recently emphasized the importance of tetraploids in speciation processes, the role of tetraploid animals in water frogs remains to be investigated.

The “mixed” animals (where genotype determination methods yielded contradictory results) seem to indicate the introgression of L- genes or genome parts into the R genome and vice versa (see Jakob 2007). Although they occur at low frequencies, they are widespread across the sampled ponds. The detection probability of such mixed genotypes will further increase with the number of microsatellite loci used for genetic investigations.

“Mosaic” animals were detectable only by flow cytometry (see Jakob 2007). They exhibit red blood cells with varying genotypic content, e.g., LLR and LRR, LLL and LLR, LL and LR. Such animals were found only rarely among adult frogs and seem to only exceptionally survive.

There were no aneuploid animals detected in our samples of juvenile and adult frogs. Aneuploid samples should be detectable with flow cytometric analysis, showing unusual broad fluorescence peaks (resulting in high coefficients of variation) or unusual relative DNA indices compared to standard cells (Tiersch and Wachtel 1993, Lowcock et al. 1997, Sharbel et al. 1997, Bihari et al. 2003).

Although rarely found, the exceptional genotypes and unusual genomic combinations demonstrate the complexity of this reproductive system and the

inherent possibility for new evolutionary pathways in pure hybridogenetic water frog populations.

There was a notable sex bias for genotypes with an excess of R-genomes (LRR, RR) to be female. This supports the theory that the male determining factors in hybrids of *R. lessonae* and *R. ridibunda* are linked to the L-genome (see Table 3). The low-frequency presence of LRR-males in some of the ponds, however, cannot be easily explained by this presumption and has to be investigated further.

The population stability was low; there was a general significant increase of the relative amount of LR-animals in Skåne over the years at the expense of LLR-animals. This trend occurred for both males and females and could be observed to continue in 2005 (D. G. Christiansen, pers. comm.). If this change in relative frequencies is due to natural fluctuations in pure hybrid populations or due to an extrinsic factor remains to be investigated with long-term studies. Due to the fact that offspring ploidy is directly determined by the parent's ploidy (triploid animals and diploid males normally produce haploid gametes, diploid females produce haploid and diploid gametes), a high percentage of triploid parents leads to a high percentage of diploid offspring, whereas a high proportion of diploid females enhance the frequency of triploid offspring. This may indicate that, in fact, transitions in relative genotype frequencies are a natural phenomenon in these populations. A study on Danish pure hybrid populations concluded that those were stable over time (Christiansen et al. 2005). The sampling regime, sample sizes, compared time spans and partly also the genotype determination methods differed greatly from our study, however, so that further analyses may come to similar results.

Östergötland

The detection of an LE- water frog system in Sweden was very surprising. Although this population lies very well in the water frog distribution range still reported in the early to mid-20th century in Southern Sweden (Gislén and Kauri 1959, Ebendal 1979), this particular population was only known to locals (Söderbäck 1984) who described the frogs simply as "edible frogs". The genotypic composition of other historically reported populations along the eastern coast of Southern Sweden remains unknown, because these populations have never been investigated on their genotypic composition (Ebendal 1979) and most probably have gone extinct until the end of the last century (Kvindall 1998, J. Pröjts in litt.). This LE-population may be considered as an intermediate link between the LL-system in Uppland and the EE-system in Skåne and could be the remnant of colonization after the last ice age. We know from Southern Sweden that selection acts against the parental forms LL and

RR. In the Baltic States, at latitudes comparable to Östergötland, the LE-system is the most widespread, showing the adaptation of this population system to such latitudes. It may well be that *R. esculenta* is at a disadvantage at higher latitudes, but there are no field or experimental data of differential larval or adult performance of different genotypes from either water frog populations north of Skåne. Further insight into the status of the Östergötland population (native vs. introduced) could be gained by genetic investigations; a first analysis with few genetic markers did deliver some support for its native status (chapter 5). In any case, this is the first discovery of a mixed *lessonae-esculenta* population system in Sweden. Besides the pond Lindalsgöl and nearby Vindommen Lake, no further localities of green frogs in the region have been found yet, so the area should be subjected to protective measures.

Acknowledgements

We would like to thank Sandra Röthlisberger for help with the lab work. The group of Rolf Zinkernagel at University Hospital Zürich provided us access to their FACSCalibur flow cytometer. Bo Baldetorp of Lund University Hospital and Eva Niederer of the Institute for Biomedical Engineering IBT at the ETH Zürich provided practical and technical advices for flow cytometric analysis. At several occasions, we had guest researchers helping us with frog catching, namely Ditte G. Christiansen, Jon Loman, Rachel Muheim, Michael Tobler, Trent J. Garner, and Stefan Goedderz.

The work was funded by the Swiss National Science Foundation, grant No. 3100-64004.00 to H.-U. Reyer. Catching permits for Sweden were provided by Länsstyrelsen i Skåne Län (522-18591-02, 522-9286-03, 522-6571-04) and by Länsstyrelsen Östergötland (522-1923-04).

Author contributions

CJ and MA contributed equally to this work. Both authors carried out all field- and lab work together. CJ performed statistical analyses and wrote the paper. CJ and MA authors discussed the results and MA and HUR commented on the manuscript.

References

Berger, L., and W. A. Berger. 1994. Persistence of all-hybrid water frog populations (*Rana kl.esculenta*) in northern Germany. Genetica Polonica **35**:73-80.

Bihari, N., M. Micic, R. Batel, and R. K. Zahn. 2003. Flow cytometric detection of DNA cell cycle alterations in hemocytes of mussels (*Mytius galloprovincialis*) off the Adriatic coast, Croatia. Aquatic Toxicology **64**:122-129.

Christiansen, D., K. Fog, B. V. Pedersen, and J. J. Boomsma. 2005. Reproduction and hybrid load in all-hybrid populations of *Rana esculenta* water frogs in Denmark. Evolution **59**:1348-1361.

Ebendal, T. 1979. Distribution, morphology and taxonomy of the Swedish green frogs (*Rana esculenta* complex). Mitteilungen aus dem Zoologischen Museum in Berlin **55**:143-152.

Ebendal, T., and T. Uzzell. 1982. Ploidy and immunological distance in Swedish water frogs (*Rana esculenta* complex). Amphibia-Reptilia **3**:125-133.

Fog, K. 1994. Water frogs in Denmark: Population types and biology. Zoologica Poloniae **39**:305-330.

Fog, K., A. Schmedes, and D. Rosenørn de Lasson. 1997. Nordens padder og krybdyr. G.E.C. Gads forlag, Copenhagen.

Garner, T. W. J., B. Gautschi, S. Röthlisberger, and H.-U. Reyer. 2000. A set of CA repeat microsatellite markers derived from the pool frog, *Rana lessonae*. Molecular Ecology **9**:2173-2175.

Gislén, T., and H. Kauri. 1959. Zoogeography of the Swedish amphibians and reptiles with notes on their growth and ecology. Acta Vertebratica **1**:196-397.

Graf, J.-D., and M. Polls Pelaz. 1989. Evolutionary genetics of the *Rana esculenta* complex. Pages 289-302 *in* R. M. Dawley and J. P. Bogart, editors. Evolution and Ecology of Unisexual Vertebrates. New York State Museum, Albany, New York, USA.

Günther, R. 1990. Die Wasserfrösche Europas. A. Ziemsen Verlag, Wittenberg.

Günther, R., and J. Plötner. 1990. Mating pattern in pure hybrid populations of water frogs *Rana esculenta* (Anura Ranidae). Alytes **8**:90-98.

Günther, R., T. Uzzell, and L. Berger. 1979. Inheritance patterns in triploid *Rana "esculenta"* (Amphibia, Salientia). Mitteilungen aus dem Zoologischen Museum in Berlin **55**:35-57.

Hedges, S. B., J. P. Bogart, and L. R. Maxson. 1992. Ancestry of unisexual salamanders. Nature **356**:708-710.

Jakob, C. 2007. Structure and dynamics of pure hybridogenetic water frog populations of *Rana esculenta* in Southern Sweden. PhD-Thesis. University of Zurich, Switzerland.

Kvindall, O. 1998. Introduktion till sårbarhetsanalyser. ArtDatabanken, SLU, Uppsala.

Lowcock, L. A., T. F. Sharbel, J. Bonin, M. Ouellet, J. Rodrigue, and J.-L. DesGranges. 1997. Flow cytometric assay for in vivo genotoxic effects of pesticides in Green frogs (Rana clamitans). Aquatic Toxicology **38**:241-255.

Mantovani, B., and V. Scali. 1992. Hybridogenesis and androgenesis in the stick-insect *Bacillus rossius-grandii benazzii* (Insecta Phasmatodea). Evolution **46**:783-796.

Muller, H. J. 1964. The relation of recombination to mutational advance. Mutation Research **1**:2-9.

Ogielska, M., K. Kazana, and P. Kierzkowski. 2001. DNA content in erythrocyte nuclei of water frogs from a pure *Rana esculenta* population in Debki (Poland). Mitteilungen aus dem Museum für Naturkunde in Berlin, Zoologische Reihe **77**:65-70.

Pala, I., and M. M. Coelho. 2004. Contrasting views over a hybrid complex: between speciation and evolutionary "dead-end". Gene **347**:283-294.

Plötner, J. 2005. Die westpaläarktischen Wasserfrösche. Laurenti-Verlag, Bielefeld.

Rybacki, M., and L. Berger. 2001. Types of water frog populations (*Rana esculenta* complex) in Poland. Mitteilungen aus dem Museum für Naturkunde in Berlin, Zoologische Reihe **77**:51-57.

Schultz, R. J. 1969. Hybridization, unisexuality, and polyploidy in the teleost *Poeciliopsis* (Poeciliidae) and other vertebrates. American Naturalist **103**:605-619.

Sharbel, T. F., L. A. Lowcock, and R. W. Murphy. 1997. Flow cytometric analysis of amphibian population composition. Pages 78-86 *in* D. M. Green, editor. Amphibians in decline: Canadian studies of a global problem, Society for the Study of Amphibians and Reptiles, Saint Louis, Missouri USA.

Sjögren, P. 1991. Genetic variation in relation to demography of peripheral pool frog populations (*Rana lessonae*). Evolutionary Ecology **5**:248-271.

Sjögren-Gulve, P. 1994. Distribution and extinction patterns within a northern metapopulatin of the pool frog, *Rana lessonae*. Ecology **75**:1357-1367.

Snell, C., J. Tetteh, and I. H. Evans. 2005. Phylogeography of the pool frog (*Rana lessonae*) in Europe: evidence for native status in Great Britain and for an unusual postglacial colonization route. Biological Journal of the Linnean Society:41-51.

Söderbäck, O. 1984. Sockna: Bilder från Åtvidaberg. 2:a upplaga edition, Bokugglan i Linköping AB, Linköping.

Som, C., and H.-U. Reyer. 2006. Demography and evolution of pure hybridogenetic frog (*Rana esculenta*) populations. Evolutionary Ecology Research **8**:1235–1248.

Tegelström, H., and P. Sjögren-Gulve. 2004. Genetic differentiation among northern European pool frog (*Rana lessonae*) populations. Herpetological Journal **14**:187-193.

Tiersch, T. R., and S. S. Wachtel. 1993. Sources of error in screening by flowcytometry for the effects of environmental mutagens. ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY **12**:37-42.

Uzzell, T. 1982. Introgression and stabilization in western palearctic species of water frogs. *in* D. Mossakowski and G. Roth, editors. Environmental Adaptation and Evolution. Gustav Fisher, Stuttgart, New York.

Vrijenhoek, R. C. 1989. Genetic and ecological constraints on the origins and establishment of unisexual vertebrates. Pages 24-31 *in* R. M. Dawley and J. P. Bogart, editors. Evolution and Ecology of Unisexual Vertebrates. New York State Museum, Albany New York, USA.

Vrijenhoek, R. C. 2006. Polyploid hybrids: multiple origins of a treefrog species. Current Biology **16**:245-247.

Wycherley, J., T. J. C. Beebee, and S. Doran. 2001. Regional accents in the Pool Frog? Development of new computer analytical techniques aids bioacoustic separation of Pool

Frog populations and may elucidate the status of Norfolk Pool Frogs. Mitteilungen aus dem Museum für Naturkunde in Berlin, Zoologische Reihe **77**:25-30.

Zeisset, I., and T. J. C. Beebee. 2001. Determination of biogeographical range: an application of molecular phylogeography to the European pool frog *Rana lessonae*. Proceedings of the Royal Society Biological Sciences, Series B **268**:933-938.

Zeisset, I., G. Rowe, and T. J. C. Beebee. 2000. Polymerase chain reaction primers for microsatellite loci in the north European water frogs *Rana ridibunda* and *R. lessonae*. Molecular Ecology **9**:1173-1174.

Table 1. Ponds sampled in 2002-2004.

			Number of sampling events		
Pond	Region	Coordinates	2002	2003	2004
001	Skåne	55°35'17"N 13°21'15"E	2	2	2
010	Skåne	55°34'12"N 13°19'37"E	2	-	-
011	Skåne	55°34'06"N 13°19'47"E	2	2	2
012	Skåne	55°34'09"N 13°19'38"E	2	-	-
014	Skåne	55°34'08"N 13°19'01"E	2	2	2
021	Skåne	55°34'09"N 13°16'42"E	2	-	-
023	Skåne	55°34'23"N 13°16'55"E	2	-	-
024	Skåne	55°34'27"N 13°16'49"E	2	-	-
032	Skåne	55°34'03"N 13°12'53"E	2	2	2
032A	Skåne	55°34'27"N 13°13'03"E	2	3	2
050	Skåne	55°29'33"N 13°08'02"E	-	-	1
089	Skåne	55°36'34"N 13°23'19"E	3	3	2
101	Skåne	55°32'51"N 13°17'04"E	2	-	-
102	Skåne	55°32'51"N 13°17'13"E	2	2	2
108	Skåne	55°33'09"N 13°16'08"E	2	2	2
108A	Skåne	55°33'11"N 13°16'09"E	2	-	-
111	Skåne	55°32'06"N 13°12'33"E	2	2	2
112	Skåne	55°32'05"N 13°12'44"E	2	-	-
123	Skåne	55°35'17"N 13°21'07"E	2	-	-
126	Skåne	55°33'59"N 13°14'12"E	2	2	2
134	Skåne	55°33'03"N 13°21'22"E	2	2	2
135	Skåne	55°33'12"N 13°21'39"E	2	-	-
137	Skåne	55°39'14"N 13°24'32"E	2	-	-
138	Skåne	55°31'32"N 12°55'45"E	2	2	2
139	Skåne	55°34'06"N 13°05'35"E	-	1	-
142	Skåne	55°35'08"N 13°06'42"E	-	-	1
147	Skåne	55°31'12"N 13°06'18"E	-	-	1
151	Skåne	55°27'03"N 13°10'17"E	-	-	1
154	Skåne	55°22'24"N 13°05'32"E	-	-	1
155	Skåne	55°22'08"N 13°26'14"E	-	-	1
159	Skåne	55°22'59"N 13°27'01"E	-	-	1
160	Skåne	55°40'01"N 13°25'48"E	-	-	1
161	Skåne	55°36'40"N 13°26'18"E	-	-	1
401	Östergötland	58°06'57"N 16°24'15"E	-	-	1

Table 2. Results for an ANOVA (PROC GLM of SAS) with the effects of pond, year, and the interaction pond*year on the main genotypes, by adults, females, males and juveniles. *P* values <0.05 are printed in bold. Number of samplings in 2002: 25, in 2003: 26, in 2004: 24 (see also Table 1).

			LR		LLR		LRR	
Sex	Effect	df	*P*	*F*	*P*	*F*	*P*	*F*
Adults	Pond	11	**<0.0001**	5.61	**<0.0001**	22.48	**<0.0001**	27.59
	Year	2	**<0.0001**	17.42	>0.1	1.94	**<0.01**	6.96
	Interaction	22	**<0.05**	2.16	**<0.05**	2.26	>0.1	1.53
Females	Pond	11	**<0.0001**	7.78	**<0.0001**	10.99	**<0.0001**	14.36
	Year	2	**<0.0001**	25.02	>0.1	0.45	**<0.01**	6.60
	Interaction	22	**<0.01**	3.59	>0.1	1.14	>0.05	1.77
Males	Pond	11	**<0.05**	2.49	**<0.0001**	9.25	**<0.0001**	7.09
	Year	2	>0.1	1.99	>0.05	2.56	>0.1	0.22
	Interaction	22	>0.05	1.79	**<0.05**	2.00	>0.1	0.43
Juveniles	Pond	11	>0.1	0.61	>0.1	1.49	**<0.01**	4.66
	Year	1	>0.05	3.96	>0.1	0.17	>0.1	1.96
	Interaction	11	>0.1	1.06	>0.1	1.42	>0.1	1.07

Table 3. Schematic table showing the offspring genotypes stemming from possible crosses in pure water frog populations in Southern Sweden, under the assumption that primary hybridizations occurred between LL males and RR females. Subscript indicate the sex of the offspring (m=male, f=female) or whether a gamete carries a female or male determining factor. Offspring types on grey background usually are inviable and die before reaching the adult stage**.**

	Males	LLR	LR	LRR
Females	Gametes	$L_{f,m}$	R_f	R_f
LLR	$L_{f,(m?)}$	$LL_{f,m}$	$LR_{f,(m?)}$	$LR_{f,(m?)}$
LR	L_fR_f	$LLR_{f,m}$	LRR_f	LRR_f
	R_f	$LR_{f,m}$	RR_f	RR_f
LRR	R_f	$LR_{f,m}$	RR_f	RR_f

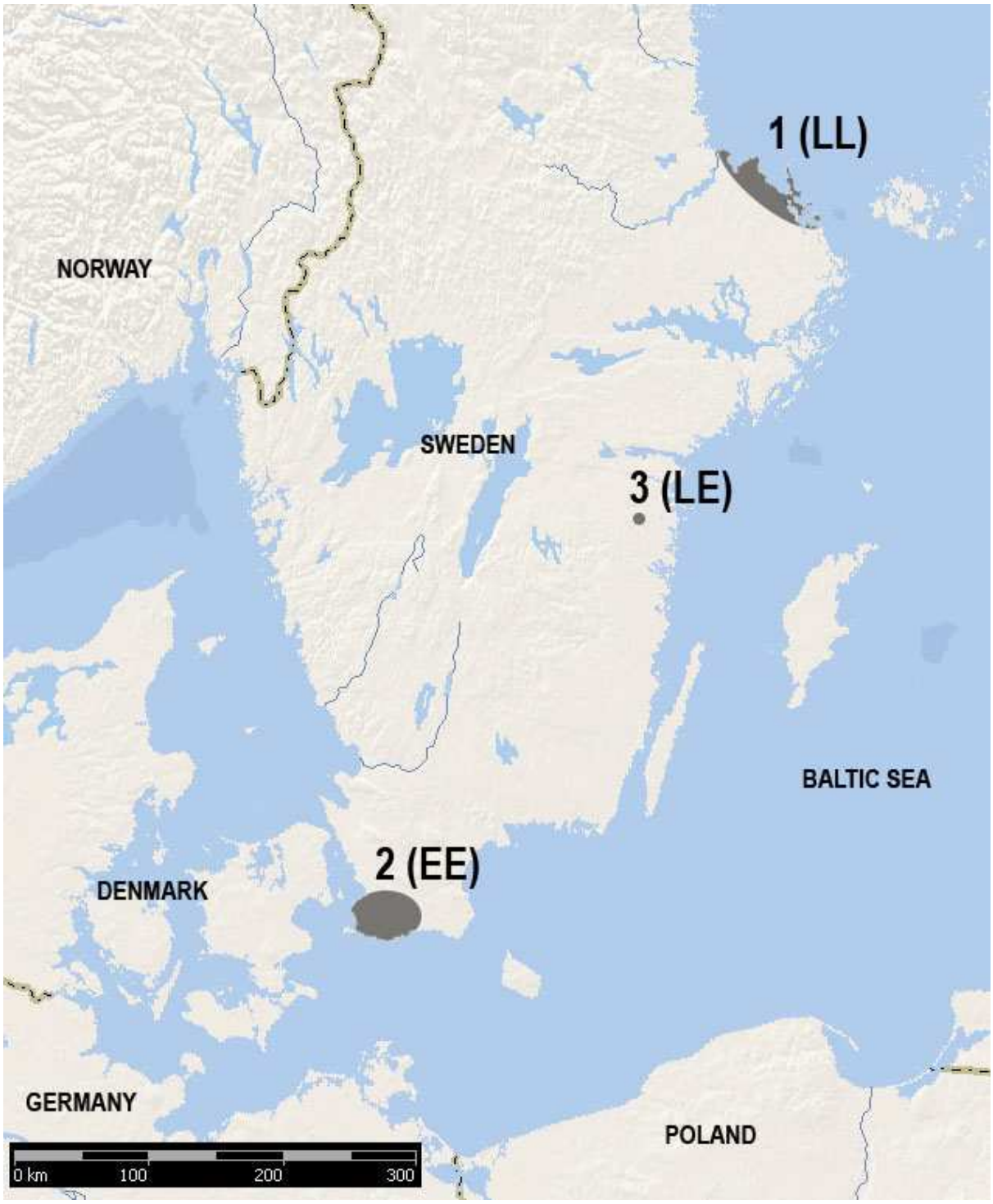

Figure 1. Map of Southern Sweden with the location of pure populations of *R. lessonae* (LL) in Uppland (1), pure hybrid water frog populations (EE) in South Western Skåne (2), and a recently discovered water frog population in Östergötland (LE, 3). Map: Adapted from Microsoft Encarta 2000 (© 1993-1999 Microsoft Corp., Redmond, WA, USA).

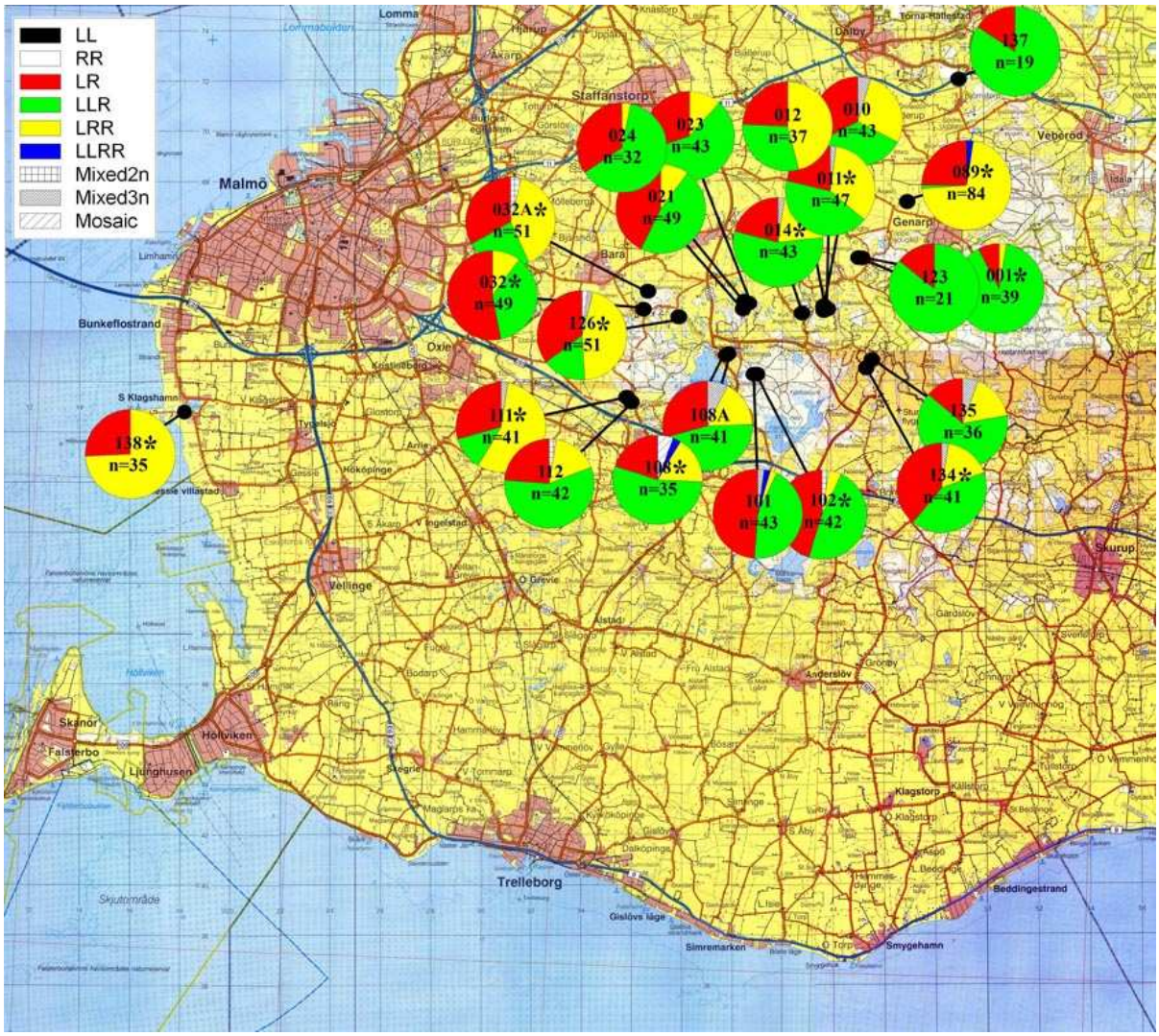

Figure 2. Genotype composition 2002 (adult frogs, pooled over all samplings). “Core ponds” are marked with an asterisk (*). Map: Adapted from Blå kartan, blad 31, edition 3 (Reproduction permission and © Lantmäteriverket Gävle 2006. Grant I 2006/1863).

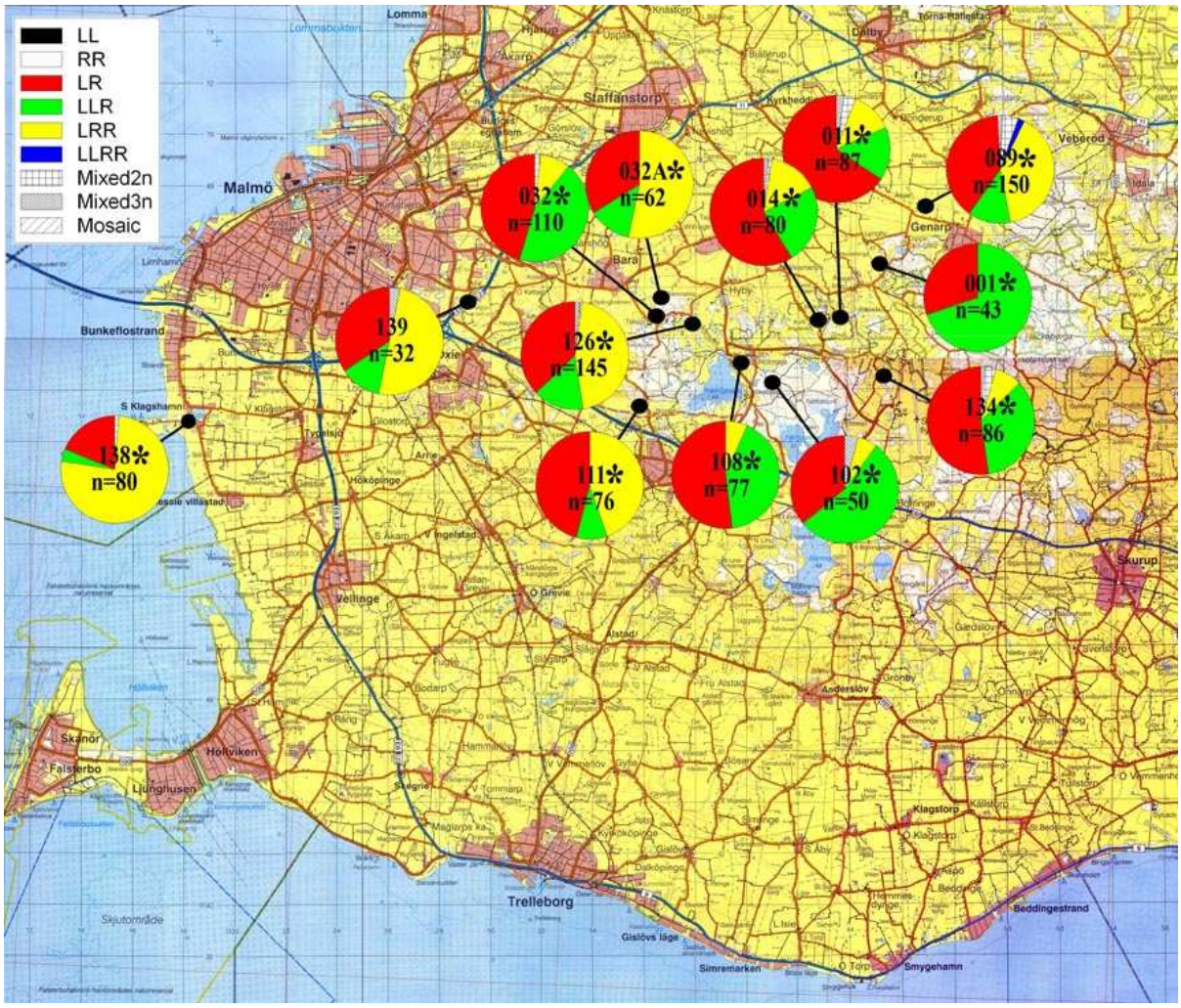

Figure 3. Genotype composition 2003 (adult frogs, pooled over all samplings). “Core ponds” are marked with an asterisk (*). Map: Adapted from Blå kartan, blad 31, edition 3 (Reproduction permission and © Lantmäteriverket Gävle 2006. Grant I 2006/1863).

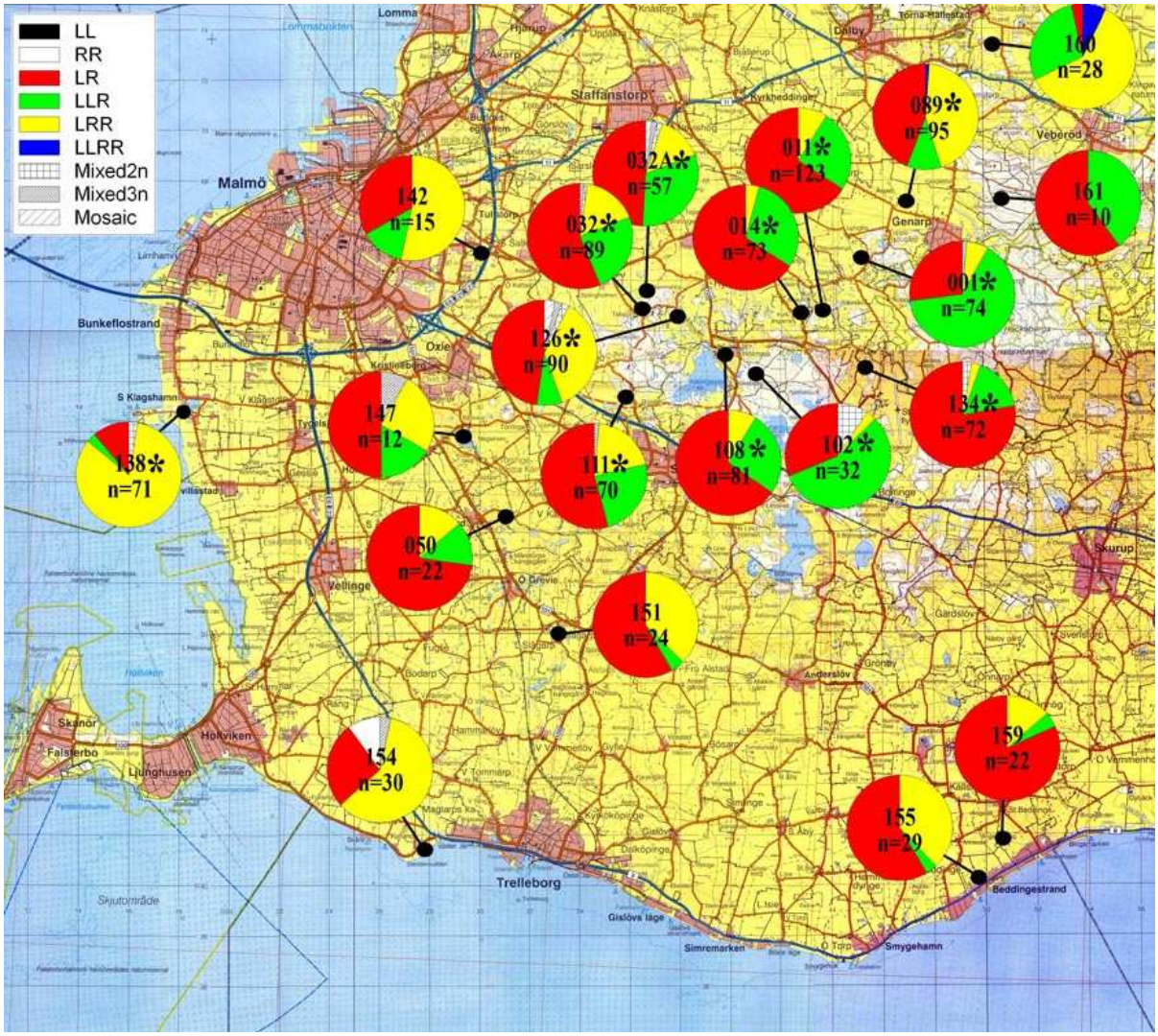

Figure 4. Genotype composition 2004 (adult frogs, pooled over all samplings). “Core ponds” are marked with an asterisk (*). Map: Adapted from Blå kartan, blad 31, edition 3 (Reproduction permission and © Lantmäteriverket Gävle 2006. Grant I 2006/1863).

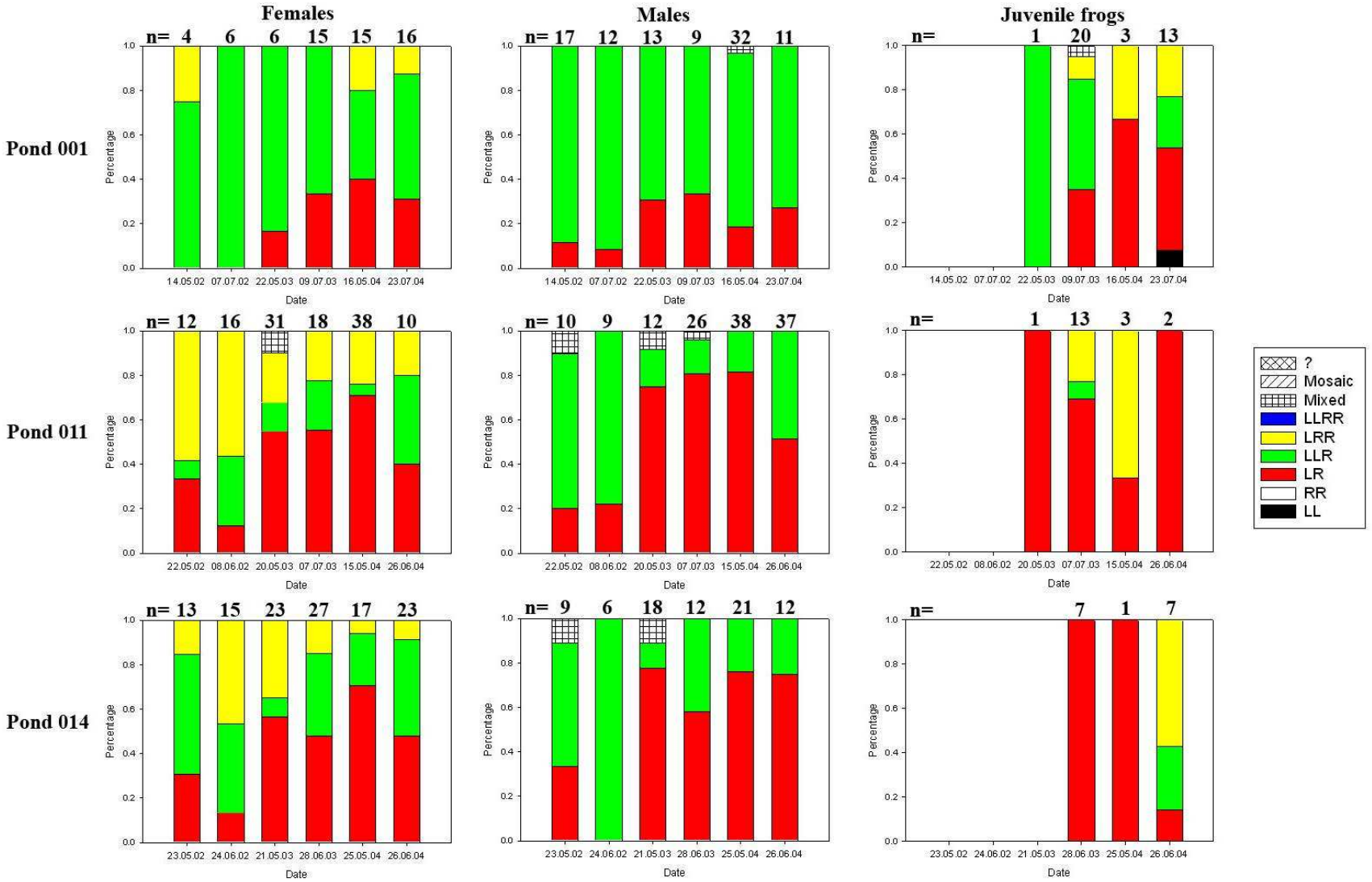

Figure 5. Genotype composition of core ponds 001, 011 and 014, listed for females, males, and juveniles. Sampling occurred from 2002-2004 twice a year; juveniles were not included in every sampling.

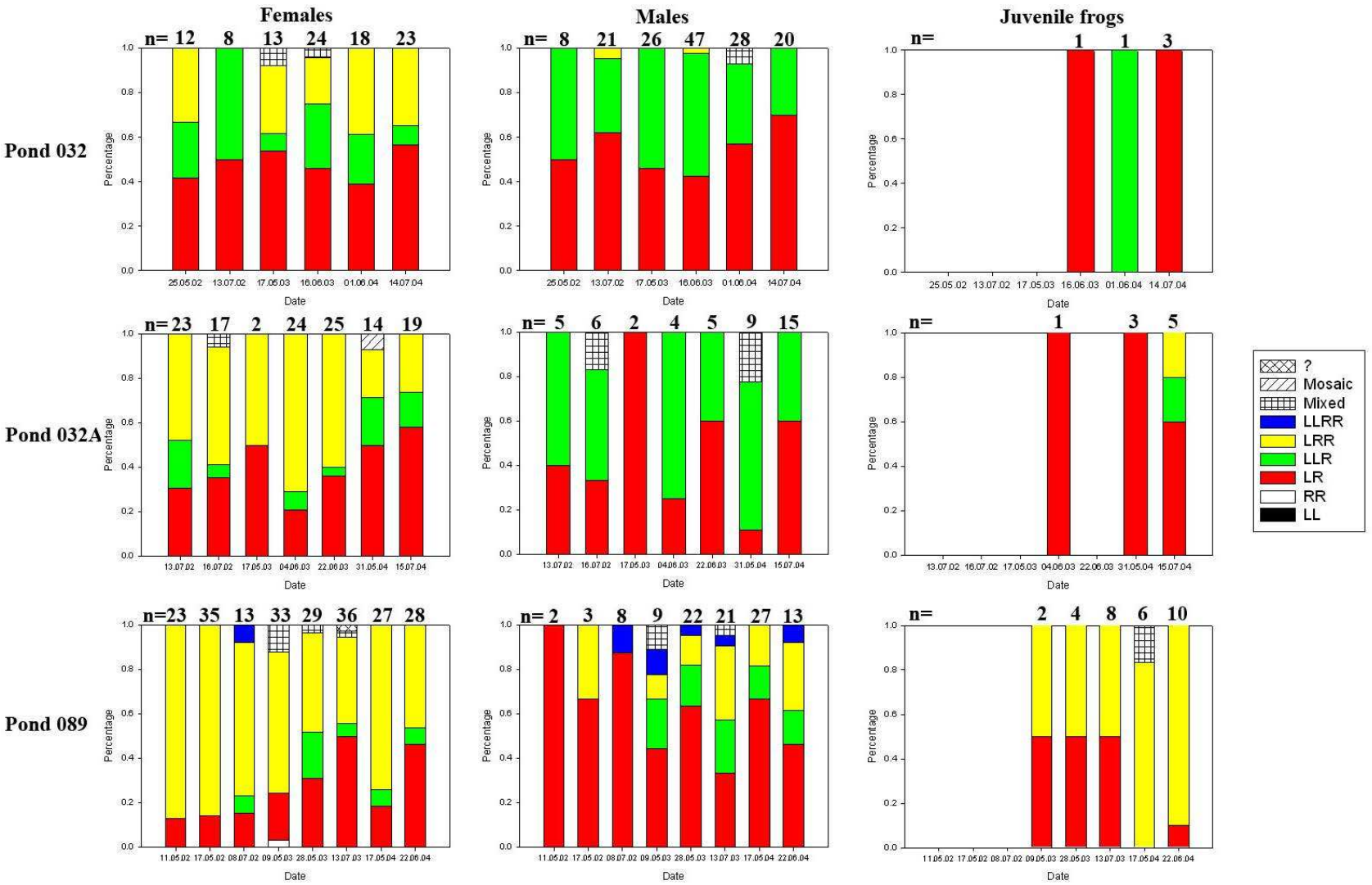

Figure 6. Genotype composition of core ponds 032, 032A and 089, listed for females, males, and juveniles. Sampling frequency: 2 (032, 032A) or 3 (089) in 2002, 2 (032) or 3 (032A, 089) in 2003, and 2 in 2004. Juveniles were not included in every sampling.

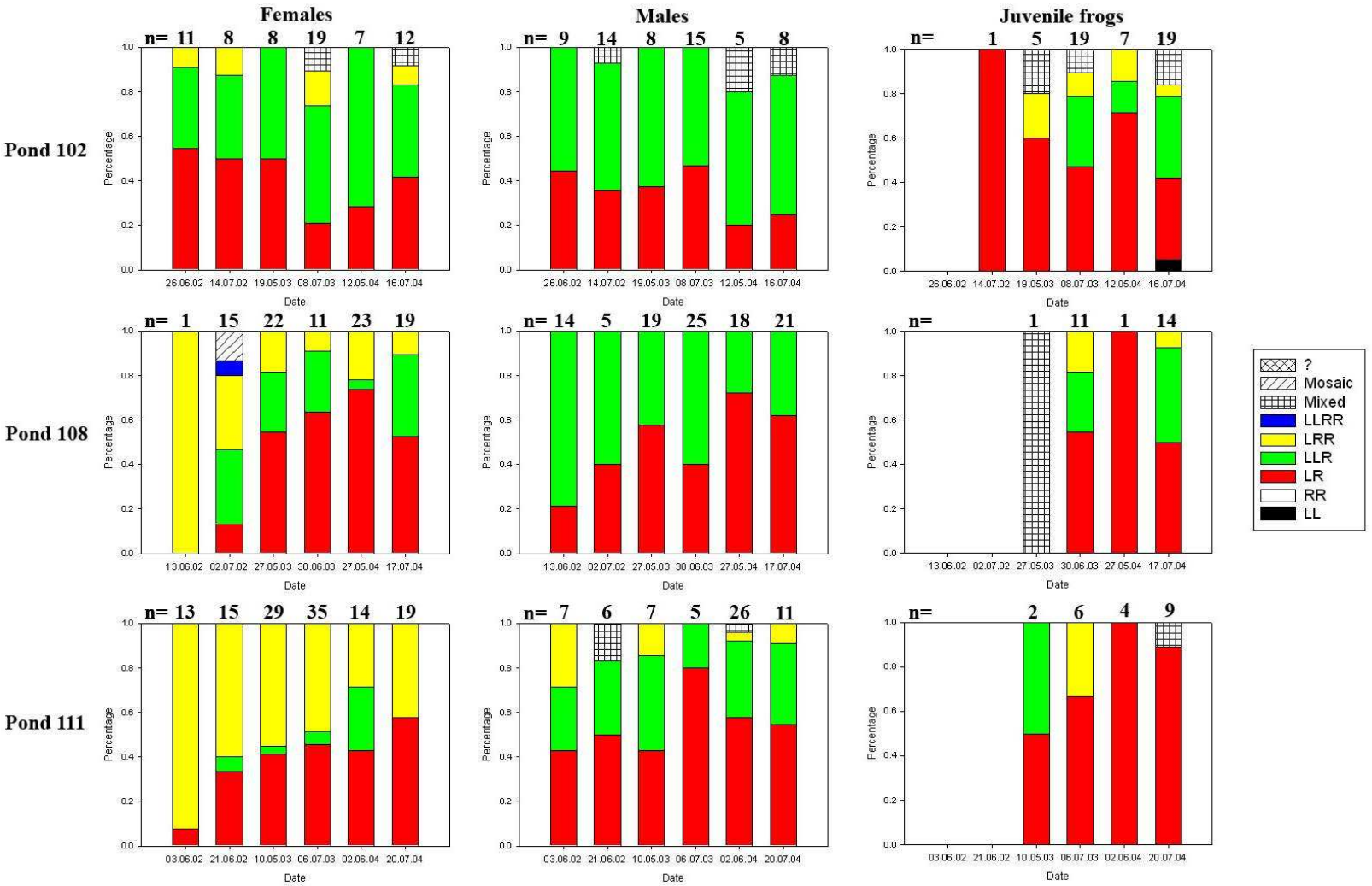

Figure 7. Genotype composition of core ponds 102, 108 and 111, listed for females, males, and juveniles. Sampling occurred from 2002-2004 twice a year. Juveniles were not included in every sampling.

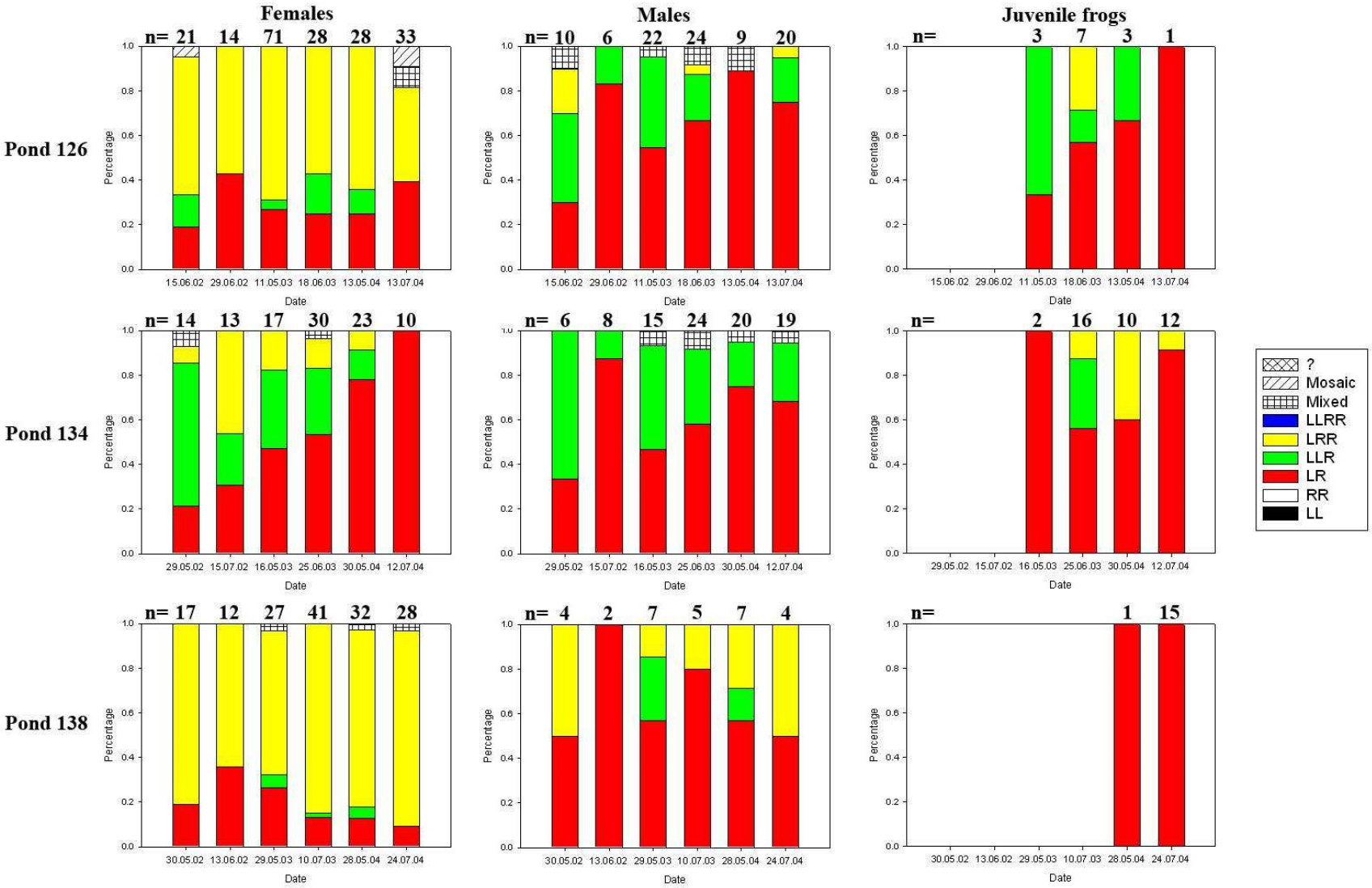

Figure 8. Genotype composition of core ponds 126, 134 and 138, listed for females, males, and juveniles. Sampling occurred from 2002-2004 twice a year. Juveniles were not included in every sampling.

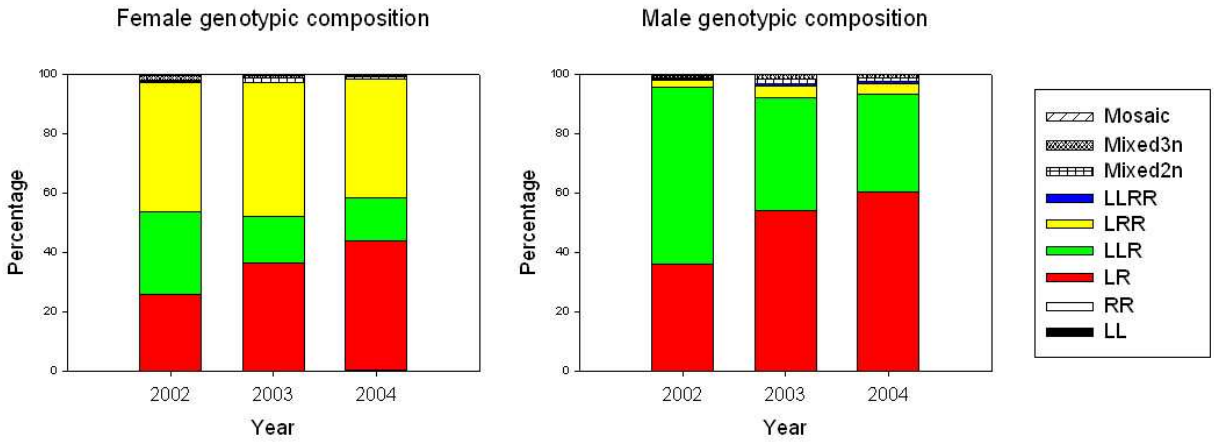

Figure 9. Relative amounts of the different genotypes in females (left) and males (right), shown for 2002, 2003 and 2004 (pooled over all ponds). Whereas the amount of LRR animals in Skåne remains stable, LR animals gain in relative frequency at the cost of LLR animals in both sexes**.**

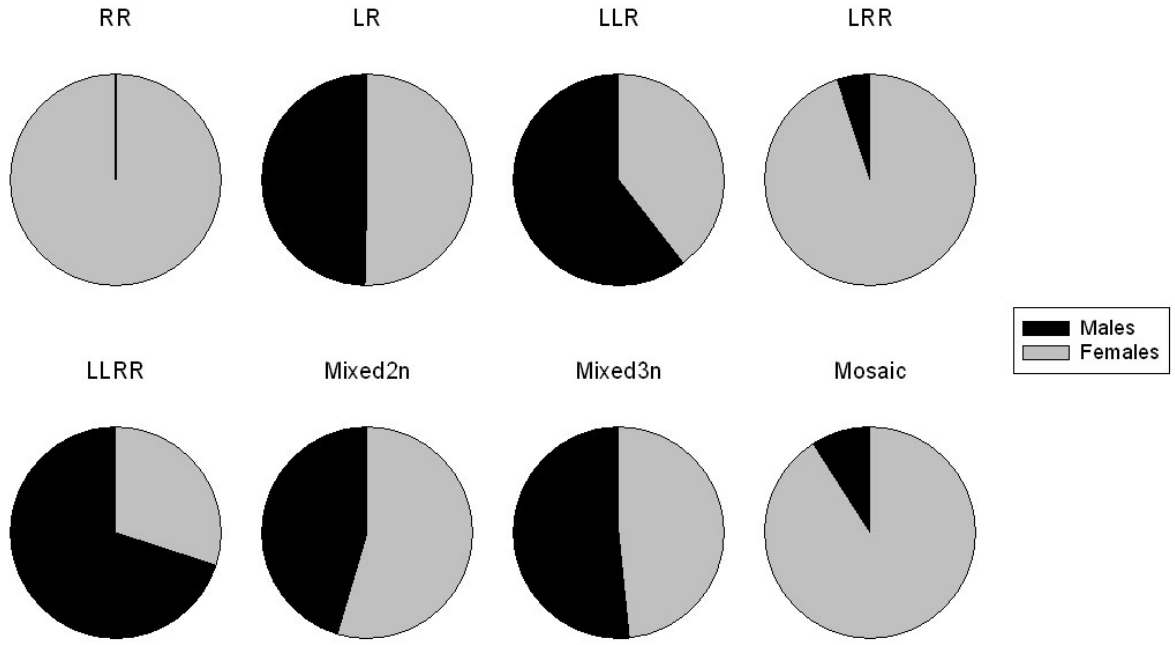

Figure 10. Sex frequencies in different genotypes (pooled over all adult individuals, 2002-2004). Whereas LRR, RR and mosaic animals are almost exclusively female (grey), males (black) are dominant in LLRR and, to a lesser extent, also in LLR.

Figure 11. Genotype composition of the Östergötland population. It clearly depicts an LE-population type, consisting only of LL and LR animals.

CHAPTER 3

Genotype composition changes during larval development in pure *Rana esculenta* populations

MARTINA ARIOLI & CHRISTIAN JAKOB

Abstract.-Hybridization between two species leads in most cases to inviable or infertile offspring due to endogenous or exogenous selection pressures. Nevertheless, hybrid taxa are found in several plant and animal genera and some of these hybrid taxa are ecologically and evolutionarily very successful. One example of such a successful hybrid is the water frog, *Rana esculenta* (genotype LR), which originated from the mating between the two species *R. ridibunda* (RR) and *R. lessonae* (LL). At the northern border of the distribution all-hybrid populations have been established, where the hybrid has achieved reproductive independence from its sexual ancestors and forms a self-sustaining evolutionary unit. Based on the gamete production of these hybrids it would be possible that parental genotypes are produced by certain mating combinations, but field sampling has clearly demonstrated that parental forms are absent among the adults.

In order to investigate potential pre- and postzygotic mechanisms that maintain such a pure hybrid system, we sampled several ponds for water frog larvae at different developmental stages. Genotype compositions were then analyzed and life-history differences between the genotypes examined. Half of the individuals in the early egg sample had a hybrid genotype, present also among the adults, the other half were parental genotypes which are not found in the adult population. The frequency of these parental genotypes decreased drastically in the later larval stage, and practically no individuals with parental genotypes were found among the metamorphs. Our finding supports the hypothesis that mating is random (i.e. no prezygotic selection) and that it is postzygotic natural selection in the ponds that acts against certain genotypes and sustains the adult pure hybrid population.

Introduction

The western group of Palearctic water frogs is a well studied complex consisting of several different “good” species which can form viable hybrid taxa. The most abundant species in Western Europe are the lake frog (*Rana ridibunda* Pallas, 1771), the pool frog (*Rana lessonae* Camerano, 1882) and the Iberian water frog (*Rana perezi* Seoane, 1885). Additionally, Uzzell and Hotz (1979) described the Italian non-hybrid (*Rana bergeri* Günther, 1985) which occurs only in Italy and resembles *R. lessonae*. There are several other water frog species in Europe, which have been studied, but not as extensively as the ones described above. In addition to the “good” species, three hybrid complexes have been described so far (Plötner 2005). Due to repeated hybridization between *R. ridibunda* (genotype RR) and *R. lessonae* (genotype LL), the edible frog (*Rana esculenta* Linnaeus, 1758, genotype LR) was formed, which is by far the most widespread hybrid taxon and distributed over most of Central Europe (Fig. 1). Another hybrid, *Rana grafi* Crochet et al., 1995, emerges from the mating between *R. ridibunda* and *R. perezi*, but this hybrid taxon occurs only in the region of the Pyrenees. The third hybrid taxon, *R. hispanica* Bonaparte, 1839, originates from the hybridization between *R. ridibunda* and *R. bergeri* and occurs only in Italy.

Possibly due to its large geographical distribution, the complex comprising *R. ridibunda*, *R. lessonae* and their hybrid *R. esculenta* has been extensively studied. The hybrid nature of *R. esculenta* was first shown by Berger (1967, 1970) through biometric analyses and breeding experiments; further investigations revealed that its reproductive mode is hybridogenetic (Tunner 1973). Hybridogenesis involves the premeiotic exclusion of one genome during gametogenesis, the clonal transmission of the other and hence, the inheritance of only one parental genome (Schultz 1969). It depends on the geographical region, which genome is transmitted (Berger 1983, Vinogradov et al. 1991) and whether genome exclusion is induced at all or not (Hotz et al. 1985, Guerrini et al. 1997). In Central and Western Europe the L-part of the genome is eliminated and, in order to restore the hybrid condition of the offspring, the hybrid has to mate with the parental species *R. lessonae*. In this area, the hybrid usually co-occurs in mixed populations with *R. lessonae* (LE-system). For the eastern part of Europe, the reverse pattern has been documented, namely that the R-genome is excluded and the L-genome is clonally transmitted to the offspring. Here, the hybrid normally coexists with *R. ridibunda* (RE-system). Because one genome is always transmitted clonally and the other genome comes from a sexual parent, the reproduction is also called hemiclonal (Dawley 1989). Vorburger (2001)

demonstrated that offspring from matings between hybrids usually do not survive due to the accumulation of mutations on the clonally inherited genome, which then occur in the homozygotic form. Therefore, the hybrid is usually forced to coexist and mate with at least one of the parental species. Models have shown that stability in these mixed systems is very sensitive to several factors, such as mating preference, female fecundity and larval performance of the involved taxa (Hellriegel and Reyer 2000, Som et al. 2000, Reyer et al. 2004).

Beside the LE- and RE-systems, all-hybrid populations of *R. esculenta* have been reported in several regions of Europe (Ebendal 1979, Eikhorst 1987, Günther 1991). Most of these pure hybrid populations are located in areas where parental species have also been recorded in populations nearby, and it can not be excluded that occasional parental migrants influence the viability of such pure hybrid populations. But at the northern border of the water frog distribution (Denmark, Sweden) there are isolated areas in which populations are presumed to have no parental forms at all (Ebendal 1979, Fog 1994, Christiansen et al. 2005). This is also true for our study area in Southern Sweden. Here, populations consist not only of diploid hybrids (genotype LR) but also of two triploid forms (LLR and LRR) and very low numbers of tetraploid frogs (Jakob 2007 & chapter 2). Although parental genotypes are supposed to be formed based on the gamete production shown in table 1, extensive sampling has revealed that no parental forms are present among adults (with the exception of one pond where 3 *R. ridibunda* females were discovered).

Little is known about the history of water frogs in this region. Ebendal (1979) reported that green frogs have been described in this area at least since around 1830 (Nilsson 1860) and that they have always been regarded as *R. esculenta* on the basis of morphometry. But because it is not always trivial to morphologically distinguish between the different water frog taxa (Pagano and Joly 1999), these early observations have to be treated cautiously. Some years later, it was confirmed by albumin electrophoresis that these Swedish water frogs are indeed diploid and triploid hybrids, but it remained unclear which of the two triploid genotypes occurs (Ebendal and Uzzell 1982).

Two scenarios about the origin of these pure *R. esculenta* populations are possible: first, only the hybrid has reached Southern Sweden or, second, parental forms have been present during the initial colonization of this area, but later were outcompeted by the hybrid. Under both scenarios the question how these populations retain pure hybrid status remains unsolved. Because all three hybrid genotypes (LR, LLR and LRR) usually co-occur in the same ponds (Jakob 2007 &

chapter 2), some matings should result in offspring of the parental genotypes (LL and RR) (Table 1). We tested the following two explanations for their absence among adults:

a) Mating is assortative and only those female x male combinations occur that lead to offspring with genotypes present also among adults.
b) Mating is random and all genotypes are present in the early stages but some are at a disadvantage (ecologically or genetically) and thus disappear during development.

To distinguish between these two possibilities, we sampled twelve ponds for water frog larvae at different developmental stages and analyzed the genotype composition and life-history differences between genotypes.

Methods

Samples and source populations

In 2003, we sampled twelve different ponds in Southern Sweden (for details see Jakob 2007) for their genotype composition at three different larval stages: egg stage, tadpole stage and metamorph stage. The aimed target numbers per pond were: 5-7 egg clutches, 25 tadpoles and 20 metamorphs. However, these sample sizes were not always achieved due to reasons mentioned in the results section; for actual sample size see table 2. Genotypes were determined from blood and tissue samples via flow cytometry and microsatellite analysis (see below). For the egg stage, we collected egg clutches in each pond at the beginning of June (June 1 – June 10) and raised a subsample of 15 individuals per clutch at Stensoffa, the field station of the University of Lund, under *ad libitum* food conditions until July 22. The upbringing of these eggs to tadpoles was necessary because analyzable amounts of blood and tissue can only be collected once the tadpoles have reached a certain size (~ 50 days old). Approximately 6 weeks after the first sampling (July 16– July 21), samples for the tadpole stage were collected from the same 12 ponds by catching a random sample of tadpoles with a dip net. Both sets of tadpoles (those raised from the eggs and those sampled from ponds) were staged for their development according to Gosner (1960) and then killed with a solution of 3-aminobenzoic acid ethyl ester methanesulfonate (MS-222, 5 g/l, Sigma A5040), because it was not

possible to obtain enough blood from living animals. Tissue was collected by cutting off part of the tadpole tail. For the metamorph stage, individuals were caught by hand between August 5 and August 12 in each of the ponds. From every metamorph we took a toe clip for microsatellite analysis and a blood sample for flow cytometry analysis. Blood was obtained by cutting the web of a hind foot and collecting the emerging drop with a heparinized capillary. Additionally, we measured snout-vent length and weight of these metamorphs to check for possible differences in development and size between genotypes. All blood samples were stored in a FRC-solution and all tissue samples were kept in 70% ETOH until lab analysis for the genotype determination was done. The following year (2004) during the sampling of the adult population in the twelve ponds we occasionally encountered juveniles (1-year old) and collected tissue as well as blood from these individuals in order to investigate the overall change in genotype proportions through the first hibernation. The number of collected juveniles ranged from 3 (pond 126) to 24 (pond 102) per pond and added up to a total of 149 juvenile frogs.

Genotype determination

It was very important for the study to correctly determine the larval genotype; we therefore combined several techniques to obtain an accurate result. We used the flow cytometry protocol described in Jakob et al. (2007) to determine the ploidy of individuals from their blood samples. Flow cytometry allows distinguishing between LR, LLR, LRR and other ploidy levels, because L- and R-genomes have different amounts of DNA (Vinogradov et al. 1991). Tissue samples were extracted using QIAamp® DNA mini kit (Qiagen). All individuals were screened for variation at seven polymorphic microsatellite loci: Ca1b5, Ca5, Ca18 (Garner et al. 2000), Res16 (Zeisset et al. 2000), Ca1b6, Re1CAGA10, Ga1a19 (unpublished, chapter 4). Two loci (Ca5, Ca18) showed only alleles for the L-genome. The other 5 loci were not species-specific, meaning that they showed alleles for both the L- and R-genome; but at all these loci the different alleles could unambiguously be assigned to either the L- or the R-genome. The microsatellite loci Ca1b5, Ca1b6, Ga1a19 and Res16 showed gene dosage (Christiansen 2005) which - in addition to flow cytometry - provided further information about the exact genotype. If flow cytometry and all microsatellite loci showed the same result, the individual was clearly assigned to one genotype. In some cases, however, the results were unusual or contradictory, even after reanalysis; e.g. flow cytometry indicated triploidy and one or several microsatellite loci showed LLR and the rest LRR. We also found cases where flow cytometry and most microsatellite loci indicated an LLR genotype, but one locus showed only LR, so

there was one allele missing. All these cases showing repeatedly contradictory results were categorized as mixed genomic individuals, hereafter called mixed. Such individuals are also, but rarely, found among adults (Jakob 2007). Therefore, we assume that they are not aneuploid animals with additional or missing chromosome fractions, because these would not survive that long. More likely do they have the same number of chromosomes (2n = 26 or 3n = 39) but not the usual composition of L or R chromosomes, e.g., LR: L = 12 and R = 14, instead of 13 each (Ogielska et al. 2004). Such a pattern can arise if irregularities and deviations from hybridogenetic rules occur during oogenesis, which was already suggested by Uzzell et al. (1975). Additionally, we were able to determine genotypes which were triploid but not hybrids (LLL and RRR) and tetraploid individuals (LLRR). Because the triploid parental types were rare (0.4% of the whole sample), we included them for the analysis in the "normal" parental genotypes (LL/RR).

Data analysis

For each of the three offspring stages (eggs, tadpoles and metamorphs) and each pond we calculated the proportions of each genotype in the sample, which were then arcsine-square root transformed before analyses. With a general linear model (PROC GLM (SAS Institute 2002-2003)) we then tested the effects of larval stage (eggs, tadpoles and metamorphs) and pond as a random factor on the proportion of the different genotypes. In a two-sampled t-test we analyzed if the proportions of LR, LLR, LRR animals differ before (metamorph sample) and after (juvenile sample) hibernation.

To examine if genotypes have different developmental rates (measured as Gosner stage, Gosner 1960), we tested with general linear models (PROC GLM (SAS Institute 2002-2003)) the effects of genotype and pond on the development of the tadpoles. The analyses were done separately for the tadpoles raised from the egg stage and for the tadpoles caught later in the ponds because their development measurements are not directly comparable. This discrepancy was due to the fact that the two groups were not sacrificed at the same time, and raising conditions for the tadpoles of the egg stage were probably more benign at the field station than for the tadpoles living in the ponds. At the metamorph stage we used snout-vent length (SVL) and weight to examine morphological differences between the genotypes and ponds and tested them in general linear models. For all GLMs we applied post-hoc pairwise-tests (Scheffé's multiple comparison procedure) to investigate which of the genotypes differed.

In order to investigate if certain genetic combinations (haplotypes) are particularly susceptible to mortality during development, translating into a change of haplotype proportions or decrease in number of haplotypes, we analyzed the genetic variance throughout development in an analysis of molecular variance (AMOVA) (Excoffier et al. 1992) as implemented in ARLEQUIN (Schneider et al. 2000).

Results

Sampling

For several reasons the aimed sample sizes were not achieved in all ponds (Table 2). In pond 032A we found neither egg clutches nor tadpoles or metamorphs, although adult frogs were numerous. The most probable explanation for the lack of offspring lies in the oxygen content of this pond, which dropped dramatically during the season to almost zero and might not have been sufficient for eggs and tadpoles to survive. The clutches that we collected in pond 138 developed poorly, resulting in only 6 surviving tadpoles. For the later stages the aimed target was successfully achieved in this pond. We found no tadpoles in pond 108, probably due to the fact that the pond is very muddy and covered with duckweed (*Lemnaceae sp*.). Tadpoles simply might have been difficult to discover, although present, because later in the season we managed to sample metamorphs in this pond. Despite enormous sampling effort in pond 102, tadpoles were rather scarce and we detected only 4 individuals. The pond is not overgrown like 108, so detection probability is high; but pond 102 has a high abundance of fish, and hence, low tadpole survival. In accordance with the rare occurrence of tadpoles we later detected only 1 metamorph in this pond. At the metamorph stage, we were surprised not to find froglets in pond 134, although we visited the pond several times (Table 2).

Genotype composition between stages (eggs, tadpoles and metamorphs)

Pooled over all ponds, the proportions of LLR and LRR hybrids did not differ between the three stages, but the LR hybrids increased significantly from the egg stage to the subsequent stages (Table 3, Fig. 2). The proportion of parental genotypes (LL and RR) significantly decreased from the egg stage throughout larval development until the metamorph stage (Table 3, Figs. 2 and 3). At the egg stage, 25% of the offspring had a RR genotype and 15% had an LL genotype. The proportion was already significantly lower during larval development (RR: 4.8%, LL: 3.3%) and only one RR

and no LL individual was found in the metamorph sample. The proportion of mixed individuals was low (6.0%) early in the development, increasing slightly at the tadpole stage (11.0%) and decreasing again in the metamorph stage (2.3%), although this change was not quite significant (Table 3, Fig. 3). Tetraploid individuals were very rare (2.8%) already at the egg stage, and none were found among the metamorph samples. The genotype analysis of the juveniles of the following year (2004) showed a similar composition as the metamorph sample of the previous year, with the exception of two discovered LL individuals (Fig. 2). When comparing the proportions of LR, LLR and LRR separately between the metamorph and juvenile sample we did the not find any significant differences (*t*-Test, df = 20, all $t \leq 1.56$, all $P \geq 0.134$).

Genotype composition between ponds

Pooled over all ponds we found no significant effect of pond on the proportion of each genotype except for the genotype LR (Table 3). This difference was mainly due to pond 102 which had no LR individual in any of the three samples, but sample size in this pond was anyway very low due to reasons mentioned above.

Differences in larval development between genotypes

The individuals that were sampled at the early stages (eggs and tadpoles) were not sacrificed at the exact same time, and the growing conditions were probably more benign for the larvae raised from the egg sample at the field station than for those in the natural ponds. Therefore, it is not meaningful to pool the developmental stage data between these two data sets.

The first sample of tadpoles that were raised from collected eggs showed overall significant differences in development (according to Gosner 1960) between the genotypes (Table 4a, Fig. 4). Pairwise comparisons revealed that offspring with the parental genotype RR (mean stage 33) did not differ significantly from LL and mixed animals (mean stages 35 and 36, respectively; both $P \geq 0.068$), but they were significantly less developed than all four hybrid genotypes (all mean stage ≥ 37, all $P \leq 0.035$). Also, LL offspring developed significantly slower than most other genotypes (all $P \leq 0.022$), except when compared to RR or mixed animals (both $P \geq 0.473$) (Fig. 4). The genotypes LR, LLR, LRR and LLRR did not differ in developmental stage for tadpoles raised from the early egg sample (all pairwise $P \geq 0.85$).

When analyzing differences in developmental stage for tadpoles randomly caught at the ponds later (tadpole stage), the overall difference between genotypes was not significant (Table 4b, Fig. 5). For tadpoles of both data sets (raised from

eggs and caught from ponds) there were significant differences in larval development between the ponds (Table 4a,b).

Later in the development, at the metamorph stage, there were only 4 genotypes present, namely LR, LLR, LRR and mixed animals. We found overall significant differences between genotypes regarding snout-vent length and weight (Table 4c, Fig. 6): individuals of the LR genotype were significantly smaller and lighter than the other genotypes (all $P \leq 0.020$) which did not differ in pairwise comparisons (all $P \geq 0.121$). Mixed individuals did not seem to be at a disadvantage in regard to size and weight compared to the other genotypes; on the contrary, they tended to be the heaviest and biggest individuals (Fig. 6). Again, ponds differed in regard to size and weight of their metamorphs (Table 4c).

Differences in haplotype frequencies

We did not detect any significant changes in haplotype frequencies between the stages for either the L- or the R- genome. For both genomes, the genetic variance in haplotypes in the sample was best explained by differences among and within ponds (Table 5).

In the L-genome we found eight different haplotypes in total, with one haplotype dominating at all three stages (70%) (Fig.7a). At the egg stage we found seven haplotypes; the tadpole sample showed all eight haplotypes, and in the metamorph sample six haplotypes were still present. Allele diversity in the R-genome was much higher than in the L-genome and resulted in a total of 27 haplotypes, but one haplotype was dominating in all three stages as well (Fig. 7b). The following numbers of haplotypes were found at the three stages; eggs: 19 haplotypes, tadpoles: 22 haplotypes and metamorphs: 19 haplotypes.

Discussion

Our results show that, in nature, all possible offspring genotypes are produced initially. Therefore, we conclude that there is no or very inefficient assortative mating acting in these ponds.

Among the first sample taken at the egg stage, half of the sample consisted of genotypes that were also found among adults (LR, LLR and LRR), but the other half was composed of unusual genotypes, i.e. those occurring among adults only rarely (LLRR and mixed individuals) or not at all, such as diploid and triploid parental

genotypes (LL, LLL, RR and RRR). The existence of LL and RR offspring suggests that the frogs do not choose their mating partners in order to avoid producing offspring with inviable genotypes, while the existence of LLL, RRR, LLRR and mixed individuals indicates that the occasional formation of unusual gametes such as diploid sperm complicates a potential mate choice system.

Assortative mating has been studied in the water frog complex before, mainly in the LE-system. In this system, it is advantageous for both taxa (LL and LR) to mate with a *R. lessonae* individual to optimize reproductive success. Abt and Reyer (1993), Roesli and Reyer (2000) and Engeler and Reyer (2001) experimentally showed that, when given a choice between LL and LR males or their calls, LR and LL females both preferred LL males. Males on the other hand did not discriminate between female genotypes, which reflects the lower male than female investment into reproduction and, hence, lower fitness costs arising from wrong matings. It has recently been shown that the lack of male choosiness can be easily explained theoretically by several factors such as overlap in size distribution of the females or relative abundance of both female taxa (Schmeller et al. 2005). In the pure hybrid populations all three occurring genotypes are very similar and definitely overlapping in their morphological appearance, especially in size (Jakob 2007). This is not surprising, considering that they all are hybrids and therefore intermediate between the parental species in their morphological features, male vocalization and other traits. Hence, it might be difficult for a frog to choose a certain hybrid genotype over another based on morphology or vocalization. To test whether discrimination is possible, choice studies for the pure hybrid systems are definitely needed, and they are presently underway (Rondinelli 2006).

The occurrence of LL and RR genotypes among the offspring can plausibly be explained by certain mating combinations alone (Table 1). However, the existence of triploid parental (LLL and RRR) and tetraploid offspring genotypes, let alone mixed individuals, is somewhat more difficult to explain. It has been shown earlier that diploid sperm can be produced in some water frog populations (Uzzell et al. 1977, Rybacki 1994, Tunner 2000), but it is assumed to be disadvantageous in terms of reproductive ability compared to haploid sperm. Jakob et al. (2007) found in a crossing experiment with Swedish hybrid frogs that the diploid males produced, among haploid L and R sperm, also diploid LR sperm. Although this seems to be the exception, it shows that hybrids can produce other gametes than the expected ones and, thus, enhance the uncertainty of the outcome when choosing a partner based on its genotype. So even if these hybrids had evolved the ability to discriminate genotypes, the outcome of mate choice in terms of the resulting offspring would

hardly be predictable. Moreover, diploid females can produce haploid and diploid eggs at the same time (Berger 1979), which leads to two very different outcomes when mated to individuals producing R sperm (RR or LRR).

In addition to potential proximate constraints on effective mate choice, there are ultimate reasons why in pure hybrid populations mate preferences are unlikely to evolve, even though Table 1 seems to suggest that individuals would benefit from avoiding matings that lead to inviable offspring, such as LLR x LLR matings resulting in LL. In a theoretical model for LLR/LR populations, Som and Reyer (2006) have recently tracked the evolutionary fate of a potential mate preference mutation. A preference for diploid LR males on the successfully propagated L gamete of a triploid LLR female would result in LLR x LR matings and produce diploid LR daughters with a preference for LR males. This is the wrong ploidy preference for all cases where LR female produce haploid eggs because it results in inviable RR offspring (Table 1). Successfully reproducing diploid LR females on the other hand produce both, diploid LR daughters that should choose LLR males and triploid LLR daughters that should choose LR males. A preference for a certain male ploidy would, thus, always be detrimental to the inclusive fitness of one of the daughter strands.

In conclusion, assortative mating in pure hybrid frog populations does not exist or work. Consequently, many different offspring genotypes are produced in natural ponds, and the absence of LL and RR among adults must be due to postzygotic selection, i.e. differential larval survival.

Among the tadpoles that were raised from collected eggs, the parental genotypes LL and RR developed significantly slower than the others. Because the raising conditions were the same for all genotypes this could hint at genetic problems which arise because two clonal genotypes with possible lethal mutations are paired and these mutations are expressed at some point during larval development. Similarly, we found in a crossing experiment done with *R. esculenta* from the same Swedish pure hybrid populations that under benign experimental conditions, LL and RR tadpoles developed slower than the other genotypes which translated into a longer time until metamorphosis and lower weight at metamorphosis. Nevertheless these parental genotypes survived in the lab at least until after metamorphosis (chapter 1). Likewise, survival in the lab until froglet stage of LL and RR offspring from a pure hybrid population was also observed by Berger (1988), but these genotypes (LL and RR) were not present in the pond at a later developmental stage. This is in contrast to results from a crossing experiment with diploid *R. esculenta* individuals from mixed LE-populations, where Vorburger (2001) showed that the resulting RR offspring from parents of the same hemiclone survived until at most 35

days after fertilization and that they usually showed severe morphological deformations. This experiment was done entirely in the lab which excludes most of the environmental selection on these genotypes and indicates that these tadpoles died due to lethal mutations which are at a homozygous state.

At later stages of the development (i.e. after tadpoles had already been exposed to the natural selection regimes of the different ponds) we found that the unusual genotypes that represented half of the sample early at the egg stage were much less abundant (only about 20%). From the decline in percentage of parental genotypes during development it is obvious that these genotypes must have a higher mortality than the others. Parental genotypes were found in all eleven ponds in the first sample, whereas among the tadpoles only five ponds still had parental genotypes. Since ponds are ecologically very different it is therefore difficult to identify specific environmental factors that cause this reduction. Under the standardized raising conditions at the field station, larvae with parental genotypes had a slower developmental rate compared to the other genotypes. In nature, such a developmental disadvantage could lead to increased mortality due to stronger competition among genotypes and/or higher predation pressure on smaller than on larger tadpoles. However, those tadpoles with the LL and RR genotypes that had survived to the tadpole stage in nature were not particularly slowed down in their development. The finding that later in development unusual genotypes were less abundant in nature compared to experimental conditions (chapter 1) clearly demonstrates that experiments alone do not give a satisfactory picture of what is actually happening in nature. At the metamorph stage there were basically only genotypes left that are also found among the adult frogs. Surprisingly, we also still found relatively many mixed individuals, which exhibited aberrant compositions of L and R chromosome numbers. These individuals seem to have no apparent disadvantage compared to the other genotypes, at least not in morphological traits. If they were true aneuploids (i.e. having missing or additional chromosomes) as suggested in other studies (Christiansen et al. 2005), we would not expect such individuals to survive so well to adulthood. There is a great similarity in genotype composition between the metamorph sample from 2003 and the juveniles from 2004, which indicates that the proportion of genotypes stayed stable over the first hibernation period.

The occurrence of unusual genotypes among the eggs in all eleven ponds confirms that the production of those offspring genotypes is not limited to just single ponds but a common phenomenon in this area. It further suggests that both genetic incompatibilities and environment are important during the selection against the

parental genotypes (LL and RR). However, samples sizes per pond are probably too small to draw conclusions about whether and how different pond conditions affect genotype shifts within a population. The result that tadpoles and metamorphs from different ponds differed in development, size and weight indicates that these ponds are variable in their environment and that these differences influence the growth of individuals independent of their genotype.

Ours is not the first study to investigate larval genotypes in all-hybrid populations; but the difficulty with the few earlier findings is that genotypes were usually identified by morphometric measures alone. Such determination, and hence, the interpretation of results, is difficult and unreliable, especially in tadpoles or small froglets and in hybrid adults where morphological measurements are often overlapping between genotypes (Jakob 2007). Morphometric measures have their limits (Pagano and Joly 1999) and even erythrocyte sizes does not always allow clear classification (Schmeller et al. 2001). Therefore, earlier studies could often not reliably distinguish between the different genotypes, especially when analyzing individuals early in the development (Eikhorst 1988). The development of water frog microsatellite markers has enabled scientists to achieve a higher resolution for genetical questions in this system (Hotz et al. 2001, Zeisset and Beebee 2003, Christiansen et al. 2005). Combined with flow cytometry, microsatellite markers enabled us to not only distinguish between the different normal genotypes, but also to detect uncommon types such as LLL or RRR, tetraploid and mixed individuals which are quite common among the early stages. With the help of microsatellite loci we were also able to examine if certain haplotypes are disappearing during development, but this was not the case. Neither did the diversity of haplotypes change during development.

Conclusion

Our study is the first to investigate the occurrence and differential survival of non-hybrid genotypes among the offspring in truly pure hybrid populations with highly reliable and exact methods. Among the eggs collected in natural ponds we found that only about half of the offspring had the genotypes commonly found among the adults (LR, LLR or LRR), the other half showed “unusual” genotypes (LL, RR, LLL, RRR, LLRR or mixed). By following the larvae through their development, we revealed that the unusual genotypes disappeared bit by bit and that at the froglet stage, the parental genotypes had disappeared. Our finding supports the hypothesis that mating is random and that selection in the pond acts against certain genotypes, so that the adult population consists of only LR, LLR and LRR genotypes. It is not yet known

which factors do impose this selection; to address this question would be very interesting for further investigations. The production of the above mentioned “unusual” genotypes seems to be a huge waste of reproductive potential. However, on a proximate level, similarities between LR, LLR and LRR and somewhat unpredictable gamete production may simply not allow the frogs to distinguish between “suitable” and “unsuitable” mating partners. On an ultimate level, selection for a preference is unlikely to evolve because, due to the genome pathways in this system, suitability constantly changes from diploid to triploid partner and back.

Acknowledgements

We wish to thank S. Röthlisberger for help with the lab work. The group of R. Zinkernagel at University Hospital Zürich provided us access to their FACSCalibur. B. Baldetorp and M. Johansson of the Lund University Hospital as well as E. Niederer of IBT at the ETH Zürich provided advice and help for flow cytometry analysis. H.-U. Reyer and G. Pasinelli provided helpful comments on earlier versions of the manuscript. This work was supported by Swiss NF grant No. 31-64004.00 to H.-U. Reyer. Catching permits for Sweden were provided by Länsstyrelsen i Skåne Län (522-9286-03).

Author contributions

M.A. and C.J. contributed equally to this work. Both authors carried out all field- and lab work together. M.A. performed statistical analyses and wrote the paper. Both authors discussed the results and C.J. commented on the manuscript.

References

Abt, G., and H.-U. Reyer. 1993. Mate choice and fitness in a hybrid frog: *Rana esculenta* females prefer *Rana lessonae* males over their own. Behavioral Ecology and Sociobiology **32**:221-228.

Berger, L. 1967. Embrional and larval development of F_1 generation of green frogs different combinations. Acta Zoologica Cracoviensia **12**:123-160.

Berger, L. 1970. Some characteristics of the crosses within *Rana esculenta* complex in postlarval development. Annales Zoologici **27**:373-416.

Berger, L. 1979. Egg size as an index of phenotype in progeny of *Rana esculenta* females. Mitteilungen aus dem Zoologischen Museum in Berlin **55**:187-202.

Berger, L. 1983. Western Palearctic water frogs (Amphibia, Ranidae): Systematics, genetics and population composition. Experientia **39**:127-234.

Berger, L. 1988. An all-hybrid water frog population persisting in agrocenoses of Central Poland (Amphibia, Salientia, Ranidae). Proceedings of the Academy of Natural Sciences of Philadelphia **140**:202-219.

Christiansen, D. 2005. A microsatellite-based method for genotyping diploid and triploid water frogs of the *Rana esculenta* hybrid complex. Molecular Ecology Notes **5**:190-193.

Christiansen, D., K. Fog, B. V. Pedersen, and J. J. Boomsma. 2005. Reproduction and hybrid load in all-hybrid populations of *Rana esculenta* water frogs in Denmark. Evolution **59**:1348-1361.

Dawley, R. M. 1989. An introduction to unisexual vertebrates. Pages 1-18 *in* R. M. Dawley and J. P. Bogart, editors. Evolution and Ecology of Unisexual Vertebrates. New York State Museum, Albany New York, USA.

Ebendal, T. 1979. Distribution, morphology and taxonomy of the Swedish green frogs (*Rana esculenta* complex). Mitteilungen aus dem Zoologischen Museum in Berlin **55**:143-152.

Ebendal, T., and T. Uzzell. 1982. Ploidy and immunological distance in Swedish water frogs (*Rana esculenta* complex). Amphibia-Reptilia **3**:125-133.

Eikhorst, R. 1987. Der Laich des Teichfrosches *Rana esculenta* Linnaeus, 1758 in einer reinen Bastardpopulation (Anura: Ranidae). Salamandra **23**:122-131.

Eikhorst, R. 1988. Die Verteilung von diploiden und triploiden Larven des Teichfrosches *Rana esculenta* in einer reinen Bastardpopulation (Anura, Ranidae). Salamandra **24**:59-68.

Engeler, B., and H.-U. Reyer. 2001. Choosy females and indiscriminate males: Mate choice in mixed populations of sexual and hybridogenetic water frogs (*Rana lessonae*, *Rana esculenta*). Behavioral Ecology **12**:600-606.

Excoffier, L., P. Smouse, and J. Quattro. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics **131**:479-491.

Fog, K. 1994. Water frogs in Denmark: Population types and biology. Zoologica Poloniae **39**:305-330.

Garner, T. W. J., B. Gautschi, S. Röthlisberger, and H.-U. Reyer. 2000. A set of CA repeat microsatellite markers derived from the pool frog, *Rana lessonae*. Molecular Ecology **9**:2173-2175.

Gosner, K. L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica **16**:183-190.

Graf, J.-D., and M. Polls Pelaz. 1989. Evolutionary genetics of the *Rana esculenta* complex. Pages 289-302 *in* R. M. Dawley and J. P. Bogart, editors. Evolution and Ecology of Unisexual Vertebrates. New York State Museum, Albany, New York, USA.

Guerrini, F., S. Bucci, M. Ragghianti, G. Mancino, H. Hotz, T. Uzzell, and L. Berger. 1997. Genomes of two water frog species resist germ line exclusion in interspecies hybrids. Journal of Experimental Zoology **279**:163-176.

Günther, R. 1990. Die Wasserfrösche Europas. A. Ziemsen Verlag, Wittenberg.

Günther, R. 1991. Europäische Wasserfrösche (Anura, Ranidae) und biologisches Artkonzept. Mitteilungen aus dem Zoologischen Museum in Berlin **67**:39-53.

Hellriegel, B., and H.-U. Reyer. 2000. Factors influencing the composition of mixed populations of a hemiclonal hybrid and its sexual host. Journal of Evolutionary Biology **13**:906-918.

Hotz, H., G. Mancino, S. Bucciinnocenti, M. Ragghianti, L. Berger, and T. Uzzell. 1985. *Rana ridibunda* varies geographically in inducing clonal gametogenesis in interspecies hybrids. Journal of Experimental Zoology **236**:199 - 210.

Hotz, H., T. Uzzell, G. D. Guex, D. Alpers, R. D. Semlitsch, and P. Beerli. 2001. Microsatellites: a tool for evolutionary genetic studies of western Palearctic water frogs. Mitteilungen aus dem Museum für Naturkunde in Berlin, Zoologische Reihe **77**:43-50.

Jakob, C. 2007. Structure and dynamics of pure hybridogenetic water frog populations of *Rana esculenta* in Southern Sweden. PhD-Thesis. University of Zurich, Switzerland.

Nilsson, S. 1860. Skandinavisk fauna. Amfibierna. 2nd ed. Gleerup, Lund.

Ogielska, M., P. Kierzkowski, and M. Rybacki. 2004. DNA content and genome composition of diploid and triploid waterfrogs belonging to the *Rana esculenta* complex (Amphibia, Anura). Canadian Journal of Zoology **82**:1894-1901.

Pagano, A., and P. Joly. 1999. Limits of the morphometric method for field identification of water frogs. Alytes **16**:130-138.

Plötner, J. 2005. Die westpaläarktischen Wasserfrösche. Laurenti-Verlag, Bielefeld.

Reyer, H.-U., M.-O. Wälti, I. Bättig, R. Altwegg, and B. Hellriegel. 2004. Low proportions of reproducing hemiclonal females increase the stability of a sexual parasite-host system (*Rana esculenta*, *R. lessonae*). Journal of Animal Ecology **73**:1089-1101.

Roesli, M., and H.-U. Reyer. 2000. Male vocalization and female choice in the hybridogenetic *Rana lessonae*/*Rana esculenta* complex. Animal Behaviour **60**:745-755.

Rondinelli, B. 2006. Female choice in all-hybrid populations of *Rana esculenta*. Unpublished MSc Thesis. University of Zurich, Switzerland.

Rybacki, M. 1994. Diploid males of *Rana esculenta* from natural populations in Poland producing diploid spermatozoa. Zoologica Poloniae **39**:517-518.

SAS Institute. 2002-2003. Version 9.1.3 SP3 for Windows. SAS Institute Inc., Cary, NC.

Schmeller, D., A. Crivelli, and M. Veith. 2001. Is triploidy indisputably determinable in hybridogenetic hybrids by planimetric analyses of erythrocytes? Mitteilungen aus dem Museum für Naturkunde in Berlin, Zoologische Reihe **77**:71-77.

Schmeller, D., R. O'Hara, and H. Kokko. 2005. Male adaptive stupidity: male mating pattern in hybridogenetic frogs. Evolutionary Ecology Research **7**:1039-1050.

Schneider, S., D. Roessli, and L. Excoffier. 2000. Arlequin ver. 2.000: A software for population genetics data analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland.

Schultz, R. J. 1969. Hybridization, unisexuality, and polyploidy in the teleost *Poeciliopsis* (Poeciliidae) and other vertebrates. American Naturalist **103**:605-619.

Som, C., B. R. Anholt, and H.-U. Reyer. 2000. The effect of assortative mating on the coexistence of a hybridogenetic waterfrog and its sexual host. American Naturalist **156**:34-46.

Som, C., and H.-U. Reyer. 2006. Demography and evolution of pure hybridogenetic frog (*Rana esculenta*) populations. Evolutionary Ecology Research **8**:1235–1248.

Tunner, H. G. 1973. Demonstration of the hybrid origin of the common green frog *Rana esculenta* L. Naturwissenschaften **60**:481-482.

Tunner, H. G. 2000. Evidence for genomic imprinting in unisexual triploid hybrid frogs. Amphibia-Reptilia **21**:135-141.

Uzzell, T., L. Berger, and R. Günther. 1975. Diploid and triploid progeny from a diploid female of *Rana esculenta* (Amphibia salientia). Proceedings of the Academy of Natural Sciences of Philadelphia **127**:81-91.

Uzzell, T., R. Günther, and L. Berger. 1977. *Rana ridibunda* and *Rana esculenta*: a leaky hybridogenetic system (Amphibia Salientia). Proceedings of the Academy of Natural Sciences of Philadelphia **128**:147-171.

Uzzell, T., and H. Hotz. 1979. Electrophoretic and morphological evidence for two forms of green frogs (*Rana esculenta* complex) in peninsular Italy (Amphibia, Salientia). Mitteilungen aus dem Zoologischen Museum in Berlin **55**:13-27.

Vinogradov, A. E., L. J. Borkin, R. Günther, and J. M. Rosanov. 1991. Two germ cell lineages with genomes of different species in one and the same animal. Hereditas **114**:245-252.

Vorburger, C. 2001. Fixation of deleterious mutations in clonal lineages: evidence from hybridogenetic frogs. Evolution **55**:2319-2332.

Zeisset, I., and T. J. C. Beebee. 2003. Population genetics of a successful invader: the marsh frog *Rana ridibunda* in Britain. Molecular Ecology **12**:639-646.

Zeisset, I., G. Rowe, and T. J. C. Beebee. 2000. Polymerase chain reaction primers for microsatellite loci in the north European water frogs *Rana ridibunda* and *R. lessonae*. Molecular Ecology **9**:1173-1174.

Table 1. Gamete production in females and males of the three hybrid genotypes and offspring types arising from the nine potential mating combinations in an all-hybrid population of *R. esculenta*. Female LR can produce both diploid eggs and haploid eggs. Genotypes in grey boxes do not occur among the adults in the population although they are initially produced (Jakob 2007).

Females \ Males	LR (R)	LLR (L)	LRR (R)
LR (LR) (R)	LRR / RR	LLR / LR	LRR / RR
LLR (L)	LR	LL	LR
LRR (R)	RR	LR	RR

Table 2. Sample sizes for three developmental stages collected from 12 ponds.

	Egg stage	Tadpole stage	Metamorph stage
Pond	No. of eggs (clutches)	No. of tadpoles	No. of metamorphs
001	51 (5)	25	20
011	51 (5)	25	25
014	52 (5)	25	25
032	61 (7)	24	15
032A	0	0	0
089	56 (6)	25	25
102	53 (5)	4	1
108	53 (5)	0	17
111	56 (5)	25	25
126	58 (6)	25	22
134	45 (5)	25	0
138	6 (4)	24	21
Total	542 (58)	227	196

Table 3. Results from a general linear model relating genotype proportions to differences between three stages (eggs, tadpoles and metamorphs) and 11 ponds (pond 032A was excluded from the analysis because no eggs, tadpoles or metamorphs could be detected).

	Stage		Pond	
Genotype	*F*	*P*	*F*	*P*
LR	7.15	**0.005**	3.95	**0.006**
LLR	2.62	0.100	1.30	0.299
LRR	0.31	0.734	1.92	0.109
LLRR	5.22	**0.016**	1.81	0.132
Mixed	3.32	0.059	1.14	0.389
LL	5.50	**0.014**	2.00	0.096
RR	7.59	**0.004**	1.22	0.341

Table 4. General linear models testing the difference in larval development (Gosner 1960) between the genotypes and the ponds at the two early stages (eggs and tadpoles) and for differences in SVL and weight at the metamorph stage.

				Developmental stage (Gosner)		Snout-vent length (SVL)		Weight	
Stage	Source	N	df	*F*	*P*	*F*	*P*	*F*	*P*
a) Eggs	Genotype	306	6	15.52	**< 0.001**	-	-	-	-
	Pond	306	9	4.31	**< 0.001**	-	-	-	-
b) Tadpoles	Genotype	144	6	1.48	0.189	-	-	-	-
	Pond	144	9	18.47	**< 0.001**	-	-	-	-
c) Metamorphs	Genotype	194	3	-	-	5.80	**< 0.001**	3.75	**0.012**
	Pond	194	9	-	-	49.28	**< 0.001**	23.37	**< 0.001**

Table 5. Variance component from an analysis of molecular variance (AMOVA) for the two genomes (L and R) in relation to the three stages (eggs, tadpoles and metamorphs) and pond within stage.

Genome	Source of variation	df	Sum of squares	% variation	*P*
L	Among stages	2	0.581	-0.82	0.780
L	Among ponds within stage	28	24.334	7.99	**< 0.001**
L	Within ponds	1068	232.459	92.82	**< 0.001**
R	Among stages	2	2.722	-1.90	0.890
R	Among ponds within stage	27	107.853	21.60	**< 0.001**
R	Within ponds	1037	399.084	80.30	**< 0.001**

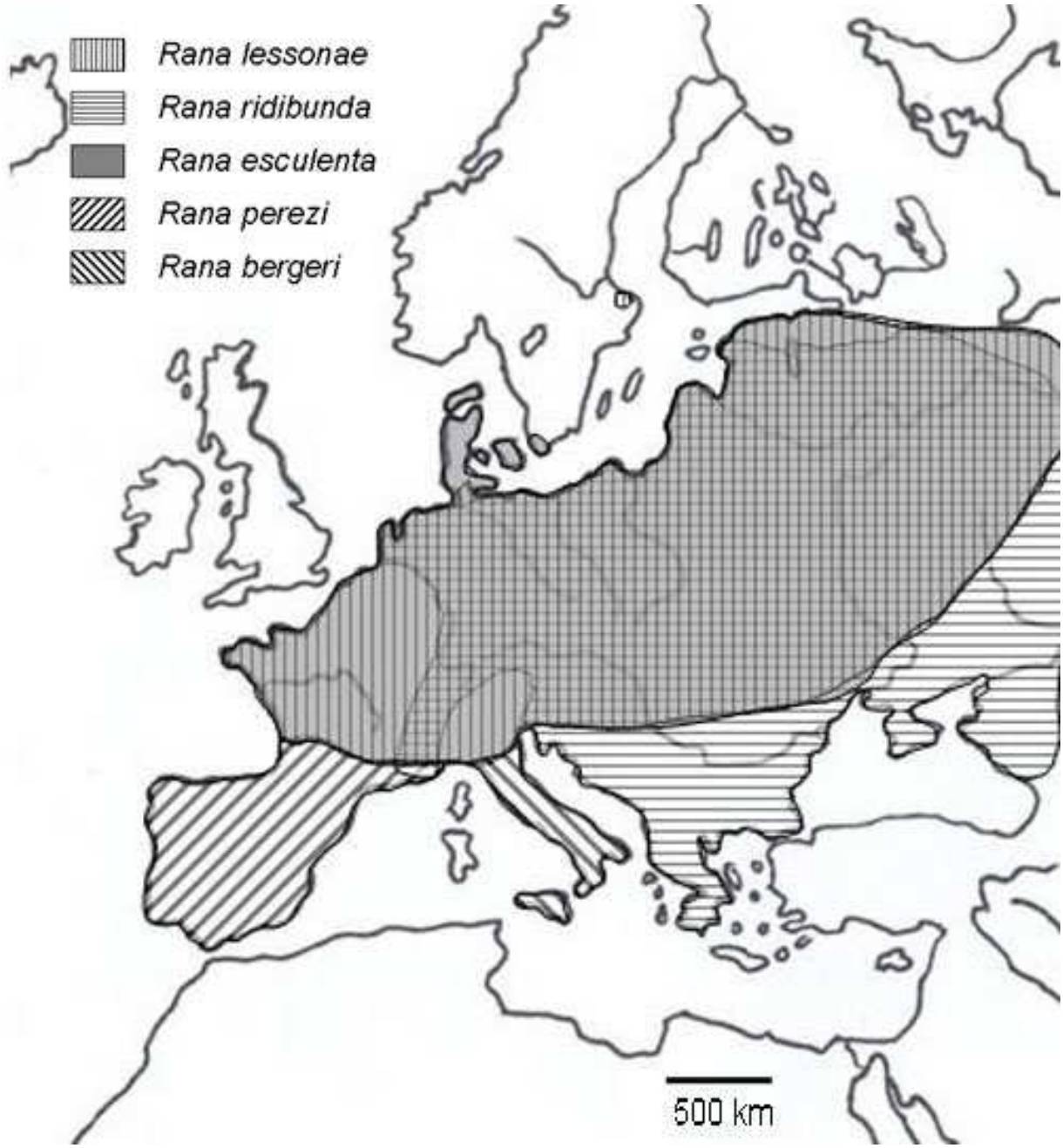

Figure 1. Map of the distribution of the Western European water frogs: *Rana lessonae*, *Rana ridibunda*, *Rana esculenta*, *Rana perezi* and *Rana bergeri*, reviewed in Günther (1990) and Graf and Polls Pelaz (1989).

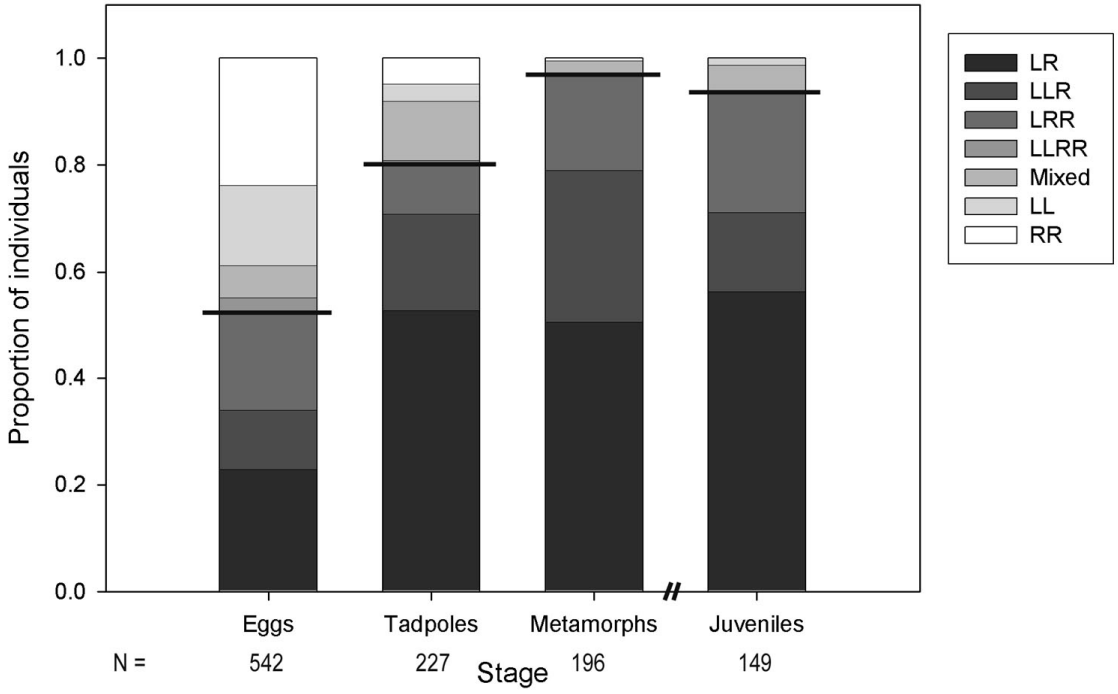

Figure 2. Proportions of different genotypes at the three stages in 2003 (eggs, tadpoles, metamorphs) and for the juveniles caught in 2004. The solid line separates the genotypes which occur commonly among adults (LR, LLR, LRR; below line) from the unusual genotypes (LLRR, mixed, LL and RR; above line).

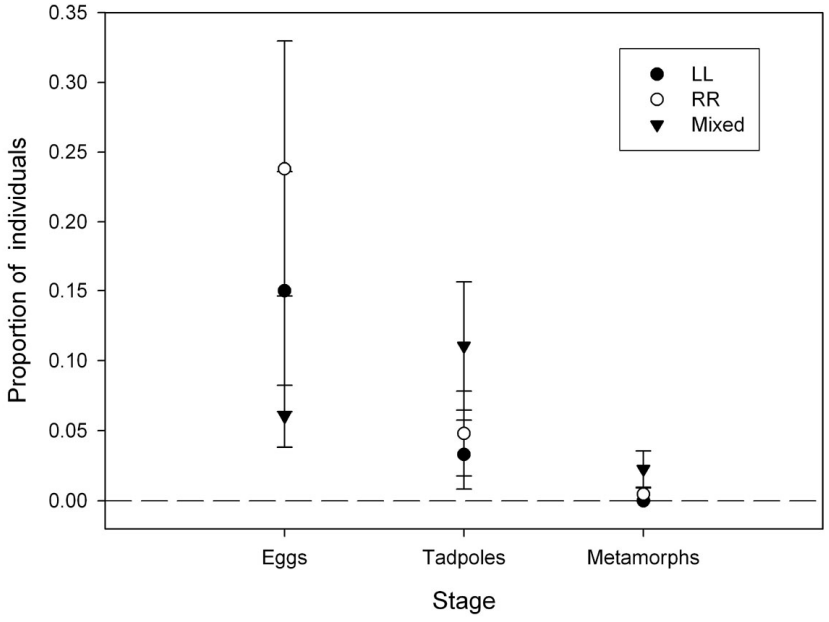

Figure 3. Proportions of LL, RR and mixed genotypes at the three stages (eggs, tadpoles and metamorphs). Shown are means ± 1 SE.

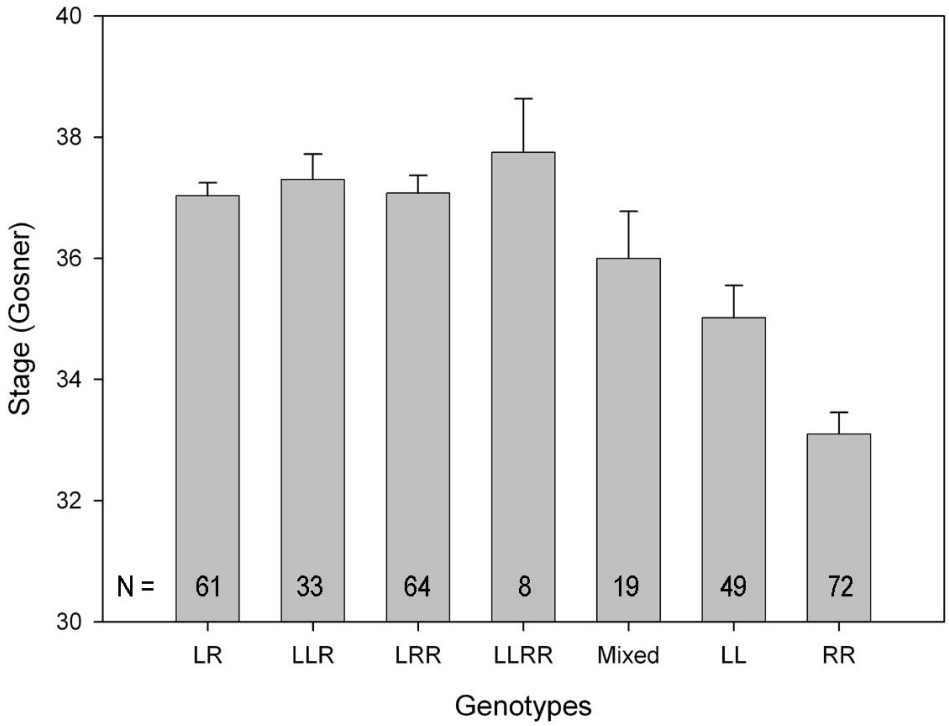

Figure 4. Differences in developmental stage between genotypes for individuals that were raised from the egg stage. Larval development was measured according to Gosner (1960). Shown are means ± 1 SE.

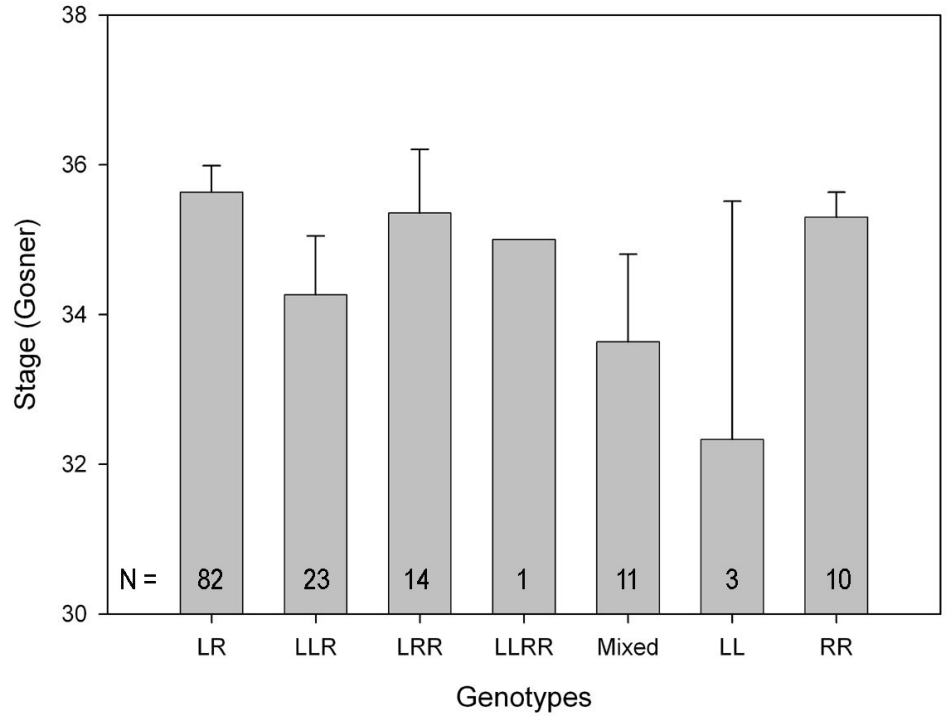

Figure 5. Differences in developmental stage between genotypes for individuals from the tadpole stage in the ponds. Shown are means ± 1 SE.

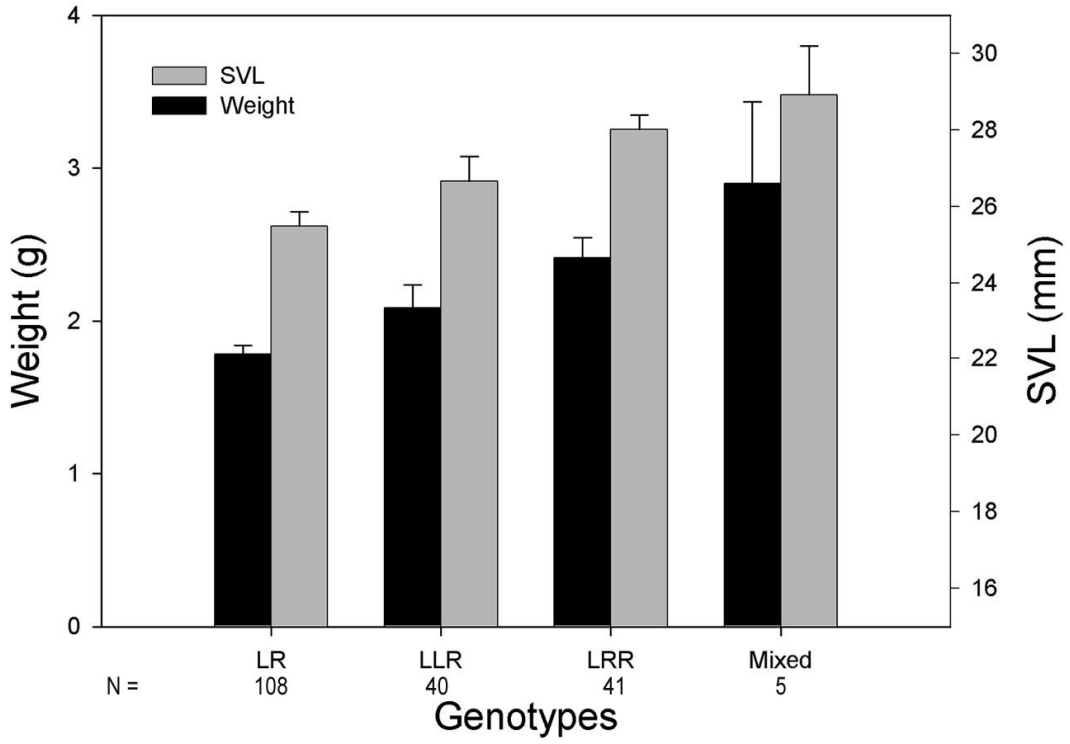

Figure 6. Differences in snout-vent length (SVL) and weight between genotypes at the metamorph stage.

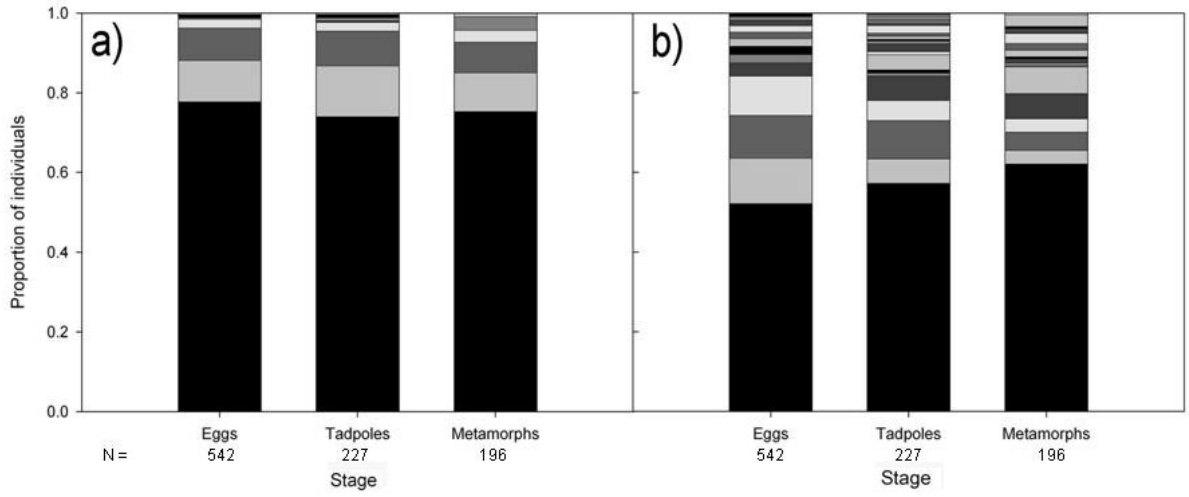

Figure 7. Proportions of haplotypes for the three different stages: a) L-genome b) R-genome. Coloration refers to different haplotypes; black indicates the most common haplotype for both genomes.

CHAPTER 4

Population genetics and diversity of the hybridogenetic water frog *Rana esculenta* in Southern Sweden

MARTINA ARIOLI & CHRISTIAN JAKOB

Abstract.-The water frog taxon, *Rana esculenta* (genotype LR), is a hybrid which originated from the mating between the two species *R. ridibunda* (RR) and *R. lessonae* (LL). Normally the hybrid is found in sympatry with one of its parental species which it depends on for successful reproduction. However, in the northern part of the distribution range pure hybrid populations can be found that have achieved reproductive independence from their sexual ancestors. These populations offer an exceptional system to study genetic diversity and its effect on the viability and structure of populations. They combine the reproductive mode of hemiclonal inheritance, usually leading to reduced diversity, with the fusion of genomes from two species, which can elevate genetic diversity considerably. We investigated the genetic diversity of 33 populations in a region in Southern Sweden in relation to their geographic location. In addition we studied the genetic population structure in this area and possible associations with ecological variables. Since the two genomes (L and R) do not recombine we investigated them in separate analyses. Among 105 tested microsatellite loci we found only seven polymorphic loci indicating that overall genetic variability is very low. However, due to its somatic hybrid status, *R. esculenta* is not suffering from direct negative fitness effects that are expected from the low genetic variability within the discrete genomes. Within the area, genetic diversity decreases from a core area to the periphery. Genetic differentiation between populations is small but significant and “isolation by distance” best explained the observed pattern of differentiation while ecological variables do not seem to influence the genetic structure.

Introduction

Genetic diversity is one of the key factors in evolution because it provides the genetic foundation for selection to act upon. The variation in genetic diversity among species or populations arises from a combination of genetic and environmental processes, such as mutation, genetic drift, gene flow or ecological changes. Nowadays we know that genetic variation can play a crucial role for the short- or long-term viability of a species or population, because it offers the potential to persist and to adapt to changing or new environments (Lande and Shannon 1996). It is also commonly acknowledged that the loss of genetic diversity may have a direct negative impact on the viability of the species or population (Amos and Balmford 2001). Single panmictic species or populations are probably rare in nature and they are mostly structured into subdivisions which again affects genetic diversity (Beebee and Rowe 2004). Natural or human caused fragmentation can reduce population size sometimes drastically which, in conjunction with the increasing risk of inbreeding in a small population, decreases their genetic variability.

But there are also mechanisms that can increase genetic variability. Although interspecific hybridization is mostly seen as maladaptive and a problem especially in conservation biology (Frankham et al. 2004), successful interspecific hybridization can instantly elevate the genetic variability in the offspring. Together with the formation of new habitats, such suddenly increased diversity even could have played an important role in rapid adaptive radiation (Seehausen 2004). However, once formed, many hybrids are no longer reproducing sexually (Bullini 1994, Dowling and Secor 1997). Vertebrate parthenogens, gynogens and hybridogens, which are of hybrid origin, pass on their genome clonally. For this clonal genome, the genetic perspective is constrained, because mutation remains the only source of genetic diversity and the accumulation of deleterious mutations through Muller's ratchet (Muller 1964) can directly limit the longevity of a clone. Yet, overall diversity in such a clonal population can be maintained through various mechanisms. One is differential selection on different clonal genomes in cases where multiple hybridization events have taken place ("frozen niche variation hypothesis"; Vrijenhoek 1984). A second mechanism operates in hybridogens, where the low diversity on the clonally transmitted genome is compensated by higher diversity on the second genome which comes from a sexual population. In some species, e.g. some fishes, genetic diversity of hybridogenetic individuals is enhanced by an incorporation of genetic material from the sympatric parental form (Pala and Coelho 2004). So, systems where a) interspecific hybridization has elevated genetic diversity, but b) clonal reproduction

limits genetic variability, are especially interesting to investigate their evolutionary potential.

The water frog complex offers a study system, where both these conditions are fulfilled. The hybrid taxon *Rana esculenta* Linnaeus, 1758 (edible frog, genome composition LR) originates from hybridization between two water frog species, the lake frog *Rana ridibunda* Pallas, 1771 (genome composition RR) and the pool frog *Rana lessonae* Camerano, 1882 (genome composition LL). Today, the two parental species are seldom found in syntopy due to different ecological requirements (Holenweg Peter et al. 2002). But it is has been demonstrated that there had been more than one primary hybridization event (Graf and Polls Pelaz 1989, Beebee 1996, Guex et al. 2002). *R. esculenta* reproduces by hybridogenesis, in which only one genome is clonally transferred to the gametes, while the other genome is discarded from the germ line before meiosis (Schultz 1969). Offspring from matings between hybrids usually do not survive, due to the accumulation of deleterious mutations on the clonally inherited genome, which then occurs in homozygotic form (Vorburger 2001). Hybrid condition is restored in each generation by fusing the clonal gametes of the hybrid with gametes from the sexual parental species whose genome is discarded. This “hemiclonal” reproduction (Dawley 1989) forces the hybrid into coexistence and mating with at least one parental species. In a way the hybrid parasitizes the parental species by “stealing” and then discarding its genome. However, as in any “parasite-host system”, the hybrid’s existence depends on the maintenance of the parental species and stable coexistence is only achieved within certain boundary conditions for mating preferences, female fecundity and larval performance (Hellriegel and Reyer 2000).

Throughout Europe, different systems of mixed populations have been found. The most common one occurs in Central and Western Europe, where the hybrid *R. esculenta* lives in sympatry with the parental species *R. lessonae* (LE-system). Other common water frog systems consist of *Ridibunda*/*Esculenta* populations (RE) and *Ridibunda*/*Lessonae*/*Esculenta* populations (RLE) (Günther 1991). One could imagine that genetic variability can be maintained in these mixed populations to a certain degree by combining genetic material from the sexual parental species with the clonal genome in the hybrid as mentioned earlier. However, in all-hybrid populations, which mainly occur in the northern region of the distribution range (Ebendal 1979, Eikhorst 1987), the hybrid has become reproductively independent from the parental forms (Graf and Polls Pelaz 1989). This was achieved by polyploidization, more specific the emergence of triploid individuals. Polyploidy often results from malfunctioning gametogenesis in hybrids (Schultz 1969, Dufresne and

Hebert 1994). The process has been studied intensively by botanist (Masterson 1994), since it is thought to be much more common among plants than in animals. It has even been argued that hybridization and polyploidization could be an important force in speciation (Soltis and Soltis 1999, Otto and Whitton, 2000).

In the water frog system, two types of triploid animals have been established, namely LLR and LRR. Triploids individuals are also found in mixed population systems in Northern Germany (Günther 1975, Eikhorst 1984), Poland (Rybacki and Berger 2001) and the Ukraine (Borkin et al. 2004), but they play a key role in the all-hybrid populations. In these pure hybrid populations, the triploid individuals take over the role that the parental species has in mixed populations, namely to act as a sexual host, mate with the diploid hybrids (sexual parasite) and thus provide them with the genome that they discarded during gametogenesis. Diploid eggs are only produced by diploid females (LR), while diploid males and triploid individuals produce haploid eggs and sperm, respectively (Table 1, chapter 1). Genomes are passed from diploids to triploids and vice versa and ploidy levels are therefore not genetically separated (Som and Reyer 2006). In several systems that combine diploid and triploid individuals, the triploids are reproducing clonally (*Poecilidae*) (Lampert et al. 2005) or apomictically (*Taraxacum* section *Ruderalia*) (Richards 1973). Hence, it is often assumed that the two ploidy types are reproductively isolated and no gene flow is present. Nevertheless, several other studies have shown that such complete isolation in these systems is not always present in nature (Menken et al. 1995, Meirmans et al. 2003).

The area of Scania (Southern Sweden) is geographically separated from the rest of the water frog distribution and it stands out because it is an area with truly pure hybrid populations. We chose this area for our study because the pond landscape is ecologically diverse, which could have led to differential selection on different clonal lineages according to the Frozen Niche Hypothesis. All three hybrid genotypes (LR, LLR, LRR) occur in mixed populations, nevertheless the ponds differ in their genotype composition (Jakob 2007 & chapter 2). Our study pursued two main goals:

1) To assess the genetic diversity of these pure hybrid populations within a defined area at the northern border of the taxon distribution and deduce possible evolutionary implications.
2) To examine whether we find genetic structuring within such a small and well defined area and, if so, to check for any associations with ecological features or genotype composition of the ponds.

Methods

Samples and Source Populations

In an area of 50x40 km in Scania, Southern Sweden, we caught a total of 2995 frogs in 33 ponds. We sampled between 19 and 130 individuals per pond per year. Individuals that were younger than two years were not sampled. Twelve ponds were sampled in all three years (2002/2003/2004). Additionally, 11 ponds were sampled only in 2002, one pond was only sampled in 2003 and 9 ponds were sampled exclusively in 2004 (see Table 2, Fig. 1)

Frogs were caught at night by hand. The following day, we took morphological measurements (snout-vent length, tibia length and callus length) and collected a blood and tissue sample (first segment of the forth toe). Tissue samples were stored in 70% EtOH until used for the microsatellite analysis. Blood was collected by cutting the web in the hind leg, drawn off with a heparinized capillary and then stored in a sucrose buffer at –50℃. The blood sample was used for flow cytometry analysis, with which we determined ploidy of the frogs. Morphology, microsatellite analyses and flow cytometry results were combined to determine the genotype of each individual. For further information about these methods see Jakob et al. (2007).

Pond features

We obtained the exact geographical positions of the ponds using the program Kartex 2.10 (Lantmäterieverket 1996). From arial pictures, provided by the Geografiska Sverigedata (GSD) we calculated the area and perimeter of each pond using ArcView GIS 3.3 (ESRI and Environmental Systems Research Institute 1992-2002). Distances between ponds ranged from 80 m (108/108A) up to 39000 m (154/160). In the field, we measured canopy cover and the amount of submerged and emerged vegetation, in order to characterize pond ecology. For canopy cover we calculated the proportion of the pond edge that had trees or bushes which could shade the pond. Less than 15% cover was classified as “open”, more than 85% was considered to be “forest” and everything in between we categorized as “forest edge” (Table 2). The amount of aquatic vegetation was estimated visually during June. For these measurements we estimated the proportion of the pond which was covered by plants below the surface (submerged) such as *Potamogeton sp*., *Ceratophyllum sp.* or *Chara vulgaris*. The same visual method was used to estimate the proportion of the pond which had water plants emerging from the surface such as *Phragmites australis* or *Typha latifolia*. Similar to the categories for canopy cover, we grouped the ponds according to the proportions of submerged and emerged vegetation into three

classes (≤ 15%, 16–84 %, ≥ 85%). Submerged vegetation can be important for tadpoles in terms of retreat from predators, whereas emerged vegetation serves adult frogs as shelter.

Molecular analysis

DNA of half a toe clip was extracted using QIAamp® DNA mini kit (Qiagen) (samples 2002/2003) or BioSprint^{TM} (Qiagen) (samples 2004).

In total, 105 microsatellite primer pairs were tested with a sub-sample of 15-34 individuals from 5 different populations in Southern Sweden. Primer sets were chosen from Garner et al. (2000) (10 loci), Hotz et al. (2001) (4 loci), Zeisset et al. (2000) (9 loci) and 12 primer sequences were kindly provided by H. Hotz and G.-D. Guex. Further primer pairs were developed after screening a mixed dinucleotide (CA, GA) and a tetranucleotide (CAGA) enriched library, following the procedures in Garner et al. (2000). 39 loci did not amplify, 50 loci turned out to be monomorphic in this subsample and were, therefore, not used for population genetics analysis. For 9 of the tested primer pairs, the scored alleles were not unequivocally attributable to one of the two different genomes (L or R) and therefore they were not applied in the analysis.

We screened for variation in all individuals at seven polymorphic loci: Ca1b5, Ca5, Ca18 (Garner et al. 2000) and Ca1b6, Re1CAGA10, Re2CAGA3, Ga1a19 (Appendix 1, GenBank Accession nos: EF121547-50). With these loci, alleles could be unambiguously assigned to either the L- or the R-genome. Loci Ca5 and Ca18 were species-specific for *R. lessonae*; locus Re2CAGA3 was species-specific for *R. ridibunda*. The other four primer pairs amplified in both the L- and R- genome (Table 3).

Primer amplification and electrophoresis for the loci Ca1b5, Ca18, Ca5, Re1CAGA10 and Re2CAGA3 were done in the ecology lab at the University of Zurich. Ca1b5, Ca18, Ca5 were amplified in a total 10 µl reaction volume containing 50-100 ng template DNA, 0.5 U Taq DNA Polymerase (Sigma), 10mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5mM $MgCl_2$, 0.01% gelatin (Sigma), 100 µM of each dNTP (Roche), 0.5 µM of both forward and reverse primer. PCR was then performed using the following conditions: 3 min at 94°C, followed by 29 cycles composed of 30 s at 94°C for denaturing, 30 s of annealing at 57°C (Ca1b5) or 58°C (Ca18, Ca5), and 30 s of extension at 72°C. We added a final extension of 5 min at 72°C and stored the product at 4°C until electrophoresis. For the locus Re1CAGA10 we used 0.5 U Hot Start Taq (Qiagen) as polymerase and the protocol was adapted accordingly with an initial denaturation of 10 min at 94°C, 35 cycles with an annealing temperature of

58℃, and a final extension for 10 min at 72℃. For the locus Re2CAGA3 we also used 0.5 U Hot Start Taq (Qiagen) as polymerase and applied a touchdown protocol (initial denaturing 15min at 94℃, then 2 cycles at each 60℃, 58℃, 56℃, 54℃, 52℃, 50℃ followed by 25 cycles at 48℃, and a final extension for 10 min at 72℃). PCR products of the loci Ca1b5, Ca5, Ca18, Re1CAGA10 and Re2CAGA3 were electrophoresed using the SEA 2000® Electrophoresis Apparatus with Spreadex®gels (Elchrom Scientific, Switzerland) and stained with SYBR® Gold nucleic acid stain (Molecular Probes, Inc.). Alleles were scored against the M3 Marker (Elchrom Scientific, Switzerland) using the Q-EL™ 330 Digital Recording and Analysis System (Elchrom Scientific, Switzerland). Two of the selected primer pairs (Ca1b6 and Ga1a19) did not work contentedly on the above system because products are run double-stranded and the occurrence of heteroduplexes complicated the accurate allele scoring. PCR amplification and genotyping of these two loci was therefore performed by Ecogenics GmbH (Zürich-Schlieren, Switzerland) using a single-stranded system (ABI Prism3100) as follows: for all 2002/2003 samples, PCR amplification was performed in a 10µl reaction volume containing 10-20ng of extracted DNA, 5µl HotstarTaq master mix (Qiagen), double distilled water, and 0.5µM of forward and reverse primers each. The forward primers were fluorescently labeled with FAM (Ga1a19 and Ca1b6). The following thermo treatment was used on a TC-412 Programmable Thermal Controller (Techne): 35 cycles with 95 ℃ for 30 s, 56 ℃ for 30 s, and 72 ℃ for 30 s. Before the first cycle, a prolonged denaturation step (95 ℃ for 15min) was included and the last cycle was followed by an 8 min extension at 72℃. For all 2004 samples, the 10µl multiplex PCR reaction contained 10-20ng of extracted DNA, 5µl 2x QIAGEN Multiplex PCR Master Mix (Qiagen), double-distilled water, and 0.75µM of forward and reverse primers each. The forward primers were fluorescently labeled with FAM (Ga1a19 and Ca1b6). The following thermo treatment on a TC-412 Programmable Thermal Controller (Techne) was used: 35 cycles with 95℃ for 30 s, 53℃ for 90 s, and 72℃ for 60 s. Before the first cycle, a prolonged denaturation step (95℃ for 15min) was included and the last cycle was followed by a 30 min extension at 60℃. The amplified products were diluted and mixed with formamide containing GENESCAN-500 (ROX) Size Standard (Applied Biosystems), and the genotype was determined on an ABI Prism3100 Genetic Analyzer using GeneScanAnalysis ®Software3.7. Scoring of the alleles of these primer pairs (Ca1b6 and Ga1a19) was done again at the University of Zurich.

Data analysis

During hybridogenesis, one genome is discarded before meiosis and only one of the parental genomes is passed on to the gametes. Therefore, no or very scarce recombination occurs between the L-and R-genome. Consequently, the parental genomes are considered to be independent, and all analyses are done on the basis of haplotypes, i.e. separately for each of the two genomes.

The genomes can occur in two different states; single-genomic if the individual has only one copy of this genome (i.e. LR, LRR for the L-genome) or double-genomic, if the individual has two copies of this parental genome and can possibly have two different alleles at this locus (i.e. LLR for the L-genome). Possible null alleles could have been detected in the single-genomic state, where missing alleles would show up; but we did not find any null alleles in our sample.

Genetic diversity (GD_i, Nei 1987) per population was calculated with ARLEQUIN (Schneider et al. 2000) and tested in a regression analysis against the proportion of LLR, respectively LRR, and the proportion of L-genomes, respectively R-genomes, per population (SAS Institute 2002-2003). This analysis is based upon the assumption that in double-genomic frogs, recombination could occur and, hence, diversity is elevated in populations with a high proportion of double-genomic frogs. Additionally, we used regression analyses to test if pond size or the geographic position of the pond within the distribution area has any influence on genetic diversity.

We analyzed genetic variation among sexes, genotypes and populations with separate analyses of molecular variance (AMOVA) (Excoffier et al. 1992) as implemented in ARLEQUIN. Genetic variation among years was analyzed using a data sub-sample containing only ponds that were sampled in all three years. Pairwise F_{ST} values (Wright 1978) between all pairs of populations were calculated and tested for isolation by distance by comparing the F_{ST} /(1- F_{ST}) matrix with the matrix of the natural logarithm of the geographical distance (Rousset 1997) in a Mantel test within ARLEQUIN. The Frozen Niche Hypothesis (Vrijenhoek 1984) assumes that different genetic clones adapt to different specific ecological niches and can so create a genetical structure. Therefore, we also tested with separate AMOVAs whether a possible genetic population structure is related to the ecological pond features that we described earlier. Among-population relationships were determined by generating an unrooted neighbor-joining tree based on Cavalli-Sforza distances using the program Populations 1.2.28 (Langella 1999) and plotted with the package PHYLIP 3.6 (Felsenstein 1993).

Results

Only polymorphic loci were suitable for the population genetics analysis and were therefore used to screen the total sample of all 2995 individuals. It is remarkable that of all the microsatellite loci which were tested and amplified in these frogs, 76% were monomorphic. Since these monomorphic loci were not included in the computation of the genetic diversity of these populations, our values would overestimate diversity if we compared our results to those of other studies. For the L-genome we found only three polymorphic loci, whereas 5 loci were found polymorphic for the R-genome (Table 3). We detected between 1 and 3 alleles for the L-genome (mean 1.67) and between 2 and 7 for the R-genome (mean 4.2) which resulted in 9 haplotypes for the L-genome and 107 haplotypes for the R-genome (Table 3).

Genetic diversity (within population variation)

Gene diversity in the L-genome was generally lower than in the R-genome (mean GD_L = 0.364, mean GD_R = 0.691). Gene diversity per population ranged from 0 to 0.603 in the L-genome and 0.051 to 0.975 in the R-genome (Table 4). In order to see if the lower mean genetic diversity in the L-genome might only reflect the fact that we had only three polymorphic L- but 5 polymorphic R-primer pairs, we repeated the comparison with a sub sample of three R-primer pairs, which were chosen based on about the same number of alleles they expressed as the L-genome. Mean genetic diversity in the R-genome using the primers Ca1b5, Ca1b6 and Ga1a19 showed a similar mean genetic diversity as in the L-genome (GD_{R_Sub} = 0.378) (*t*-Test, *P* = 0.804).

Genetic diversity was not related to the proportion of triploid individuals (LLR, LRR) or haploid genomes (L, R) in the populations for either the L- or the R-genome (N = 33, all $r^2 \leq 0.077$, all $P \geq 0.118$). Pond size was also not significantly related to genetic diversity within ponds (N = 33, L: r^2 = 0.097 *P* = 0.077; R: r^2 = 0.000 *P* = 0.984).

However, we did find that genetic diversity in the R-genome depended on the location of the pond within the distribution area: it decreased significantly from the centre to the periphery (N = 33, r^2 = 0.512, $P \leq 0.001$; Fig. 2). For the L-genome, the corresponding regression is not significant (r^2 = 0.076 *P* = 0.120), although ponds closer to the centre also tend to have a higher genetic diversity (Fig. 2).

Differences between years in the core ponds (AMOVA)

In order to see if the genetic composition is changing between years, we first tested for genetic differences in the 12 core ponds between the three years. The AMOVA showed that the years were not significantly different for either the L-genome (-0.33%, P = 0.951) or the R-genome (-1.20%, P = 0.990) (Table 5). Therefore, all populations were used to test for genetic structuring within this area.

Differences between genotypes and sexes (AMOVA)

To investigate if allele transfer between the genotypes is unrestricted we analyzed if any of the genetic variation can be explained by the different genotype groups (LR, LLR and LRR). Our results showed that in fact genotypes differed genetically significantly; in the L-genome the genotype differences accounted for 0.36 % (P = 0.001) of the total variation, while in the R-genome 1.05% (P < 0.001) of the total genetic variation was assigned to the difference between genotypes (Table 5). Subsequent pairwise comparisons show that, with respect to the L-genome, LRR individuals are mainly different from the LLR and LR individuals, whereas with respect to the R-genome the diploid LR individuals are different from the two triploids (LLR/LRR). Males and females did not differ genetically in the R-genome (-0.20%; P = 0.877). Although marginally not significant for the L-genome, females and males seem to have restricted gene flow (0.19%; P = 0.054).

Population structure (genetic distances between populations)

Pooled over all loci which amplified the L-genome, genetic differentiation between populations was highly significant (5.86%; P < 0.001, F_{ST} = 0.059) and correlated positively with geographic distance (Mantel test, R^2 = 0.344, Fig. 3a). Pairwise F_{ST} values ranged from 0 to 0.564. In the R-genome, 18.53% of the variation was assigned to between-population variation (P < 0.001, F_{ST} = 0.185) and pairwise F_{ST} values ranged from 0 to 0.842. When tested for isolation by distance, we also found a significant positive correlation between genetic differentiation and geographic distance (Mantel test, R^2 = 0.356, Fig. 3b)

If genotypes are genetically different and the genotype composition follows a geographic pattern, it is possible that the above result of isolation by distance is also influenced by the genotype composition of a pond. Consequently, we also tested three matrices, containing the differences in the genotype proportion of LR, LLR and LRR between ponds against the genetical differences of these ponds (Mantel test). For the genotype matrices of LLR and LR we found no correlation (all r^2 < 0.103 and P > 0.102), but in the case of LRR there was a marginal significant correlation for

both genomes (L: P = 0.049, R: P = 0.040). This means the bigger the difference in proportion of LRR, the bigger is also the genetic difference between these ponds.

When tested for genetic structuring based on ecological features we did not find any significant result. The amount of canopy cover had no influence on the structuring (L: -0.12%; P = 0.381, R: 0.93%; P = 0.165), nor had the amount of submerged (L: -0.80%; P = 0.991, R: -2.11%; P = 0.984) or the proportion of emerged vegetation (L: -0.07%; P = 0.353, R: -0.61%; P = 0.545).

The effect of pond location, rather than environmental conditions on genetic structure is also visible in the consensus tree (Fig. 4). Here, populations are not clustering according to ecological variables, but rather geographically. Genetic differentiation is lower among ponds within the central and the peripheral area, respectively, than among ponds between the two areas.

Discussion

Genetic diversity

It was difficult to obtain multiple variable microsatellite markers for the analysis, and the ones that were finally used showed relatively low variability. Considering the fact that the study area is located at the northern edge of the distribution range, this is not surprising (Seppä and Laurila 1999). It has been shown in other species, that genetic variability decreases with increasing distance from the refugium during the Pleistocene (Merilä and Baker 1996, Beebee and Rowe 2000, Zeisset and Beebee 2001, Garner et al. 2004, Palo et al. 2004). Especially in anurans, it seems to be a common pattern that peripheral populations exhibit lower genetic diversity than central ones (Rowe et al. 1999, Garner et al. 2003, Garner et al. 2004). This pattern repeats itself at a smaller spatial scale within our study area. We found a core area in which population diversity is higher than in the periphery at least for the R-genome. It is known that water frogs have been around in this area at least since they were first described by Carl von Linné in 1758, but nothing is known about their exact distribution then. Based on our results we suspect that the distribution of *R. esculenta* has been restricted to a core area in former times and has expanded its range in Southern Sweden.

Low genetic diversity has usually an impact on the viability of such a genetically depleted population. Reduced variability lowers a population's ability to react to novel challenges. Additionally, inbreeding effects and genetic drift can cause extinction of a

population (Amos and Balmford 2001). Recently, several studies have tested the hypothesis that low genetic variability (low heterozygosity) in amphibian species is directly correlated with fitness measurements. But results are somewhat ambiguous. Some studies confirmed such a positive correlation (Hitchings and Beebee 1998, Rowe et al. 1999, Lesbarrères et al. 2005), others did not find any correlations (Rowe and Beebee 2001).

Sjögren (1991) and Tegelström and Sjögren-Gulve (2004) found in an isolated northern metapopulation of *R. lessonae* that genetic diversity is extremely low compared with Central European populations. They deduce that this low variability is probably a consequence of the local turnover in this metapopulation. But, other than expected, these populations showed no signs of reduced viability. Similarly, we have found in our study population that variability in each genome was relatively low. Yet, it does not seem that *R. esculenta* is suffering any negative fitness effects. Already Ebendal (1979) mentioned that the species in this area has extended its range eastwards during the 1970's, and current sampling in Scania attest to a bigger distribution than has been described before (Jan Pröjts, personal communication).

A crucial advantage for *R. esculenta* is its hybrid origin with the resulting mix of two parental genomes. Although, reproductively speaking, *R. esculenta* is clonal, somatic heterozygosity is restored every generation. As a result, individual frogs profit from an elevated genetic diversity through the combination of two (LR) or three (LLR, LRR) parental genomes – even when each genome alone has low variability. However, when occurring in homotypic form (LL or RR), resulting from hybrid x hybrid matings, this low variability within genomes could have highly negative fitness consequences.

When comparing the same number of polymorphic loci in the two genomes, there is no difference in mean gene diversity. Already Slate and Pemberton (2002) caution that sample size and number of typed loci can have a strong effect on measuring genetic diversity and, therefore, also its association with fitness. So it seems difficult to infer conclusively from our study that in this area the L-genome is less diverse than the R-genome.

Genetic diversity per population within the two discrete genomes was not related to the proportion of the respective genome in these populations (%L or %R) nor with the proportion of the heterozygote genotypes in these populations (%LLR or %LRR). So far it has never been explicitly tested if a genome has the possibility of recombining when in a double-genomic individual (i.e. L-genome in an LLR frog or R-genome in an LRR frog), although it is often suspected by authors (Günther 1983, Eikhorst 1988, Christiansen et al. 2005). For triploids, it is assumed that the genome

that is only present once is eliminated and the two remaining genomes segregate according to normal meiosis (Günther et al. 1979, Vinogradov et al. 1990). Our data do not support the hypothesis that diversity is elevated in populations where recombination in many double-genomic individuals is possible. But it does not exclude such a possibility completely either, because if there is only little diversity present at the beginning in such a population, we would not detect any occurring recombination.

Actual population size is not always easy to measure, but it tends to be positively correlated to habitat size. Due to increased ecological diversity in bigger habitats, one could expect habitat size to have a positive influence on genetic diversity. But unlike other studies (Frankham 1996, Michels et al. 2003) we found no such association of pond size with genetic diversity in either one of the genomes.

Genetic differences between sexes and genotypes

We did not detect differences between the sexes in genotypic composition. In some Central European populations, *R. esculenta* is strongly sex-biased, for example at Neusiedlersee, Austria (Tunner 1974), where hybrids are almost purely female, or in Latvia (Borkin et al. 1986) and near the Oder river, Germany (Uzzell et al. 1977), where populations with only male hybrids were recorded. In the Swedish populations we found almost no LRR males (Jakob 2007 & chapter 2). This is probably due to the usual initial hybridization event between *R. lessonae* males and *R. ridibunda* females with the result that there are no male-determining factors on the R-genome. Haplotypes within genomes, however, do not seem to be sex-linked in the examined Swedish populations and there are apparently no barriers for gene flow between males and females. Therefore, it is valid to analyze both sexes together for the population structure.

The three genotypes differed in their genetic composition in both genomes. This is difficult to explain, because both genomes “travel” between genotypes (Fig. 5). An L-genome is usually inherited between LR and LLR, once it is in an LRR individual, it is not passed on. Conversely, the R-genome is passed on mainly between LR and LRR and if it is in a LLR frog, it is at a dead end. Our data show that, with respect to the L-genome, the LRR individuals are genetically slightly different from the LLR individuals. This might reflect unidirectional gene flow due to the fact that L-genomes get eliminated from the gene pool once they are in an LRR frog. With respect to the R-genome, LR individuals are genetically different from the other two genotype groups (LLR, LRR). This is surprising because LR individuals apparently form the link between the two triploid types. When comparing the two genome pathways, we find

that, theoretically, the L-genome is more often lost from the gene pool than the R-genome. We have to consider though, that the genotype composition in a population is rarely balanced and the number of transmitting pathways strongly depends on the mating pattern or (if mating is random) on the exact proportion of each genotype in a particular pond.

Temporal and spatial genetic differentiation

It was not possible to sample all populations in the same year; therefore, we tested for annual variation in the 12 core ponds that were sampled in each of the three years. These twelve ponds were not different in their genetic composition between these three years; thus, we could eliminate the possibility that variation between ponds was due to the different sampling years. In many studies, population samples are taken at different times, due to logistical reasons, but temporal variation is only seldom accounted for, although it can influence results on population structure markedly as, for instance, shown in marine fish (Dannewitz et al. 2005). If part of the variation is due to temporal difference, this can confound geographical structuring in regard to genetic variation.

Most studies on genetic structuring within anuran populations have focused on large scale settings (Rowe et al. 1998). Only recently has the interest in fine scale studies increased in connection with dispersal barriers and possible inbreeding effects for amphibian populations. The findings of these studies are not always in agreement, but it has to be noted that they comprise a wide set of different species (Seppä and Laurila 1999, Rowe et al. 2000, Tallmon et al. 2000, Lampert et al. 2003). Most amphibians are thought to have limited mobility and to express high site fidelity, inferring that they are bad dispersers (Beebee 2005). This argues for population structuring even over short distances. In our study populations of *R. esculenta*, we could show a significant population differentiation for both genomes on a rather small landscape scale. However, the variation explained by population differences was much smaller for the L-genome than for the R-genome. The best explanation for the structuring among these *R. esculenta* populations was the geographic distance between ponds. Such isolation by distance has also been demonstrated in other studies on small scale (Lampert et al. 2003) or on large scale (Palo et al. 2004), but distance effects were absent in a study by Seppä and Laurila (1999). The pattern of “isolation by distance” needs time to evolve and arises through the balance of local genetic drift within a population and dispersal of individuals between populations. We know through Carl von Linné that *R. esculenta* has occurred in this region at least since 1758, so there was enough time for such a

pattern to develop. Although the area of Southern Sweden is quite populated and agriculture shapes the landscape, there seem to be no major barriers, such as streets or rivers that negatively influence dispersal of the frogs over small distances. *R. esculenta* has been shown to migrate or disperse over long distances within or between seasons (Holenweg Peter 1999) or to their hibernation site (Tunner 1992). But in order to have an input on the genetic structure, the disperser has to actually reproduce in the new population and this is somewhat more difficult to test.

We found that none of the ecological variables (canopy cover, submerged and emerged vegetation) explained the genetic structure in this area. Therefore, we infer that either the realized niches of the genotypes are not adequately represented by these variables or that the area does not contain different frozen clones which are adapted to a specific habitat; rather, the hybrids may possess generalist genotypes which can deal with a variety of habitats. Where ecological measurements have been shown to be important explaining differences in amphibian species composition in ponds (Holenweg Peter et al. 2002, Plenet et al. 2005, Van Buskirk 2005) they explained differences between species rather than genotype differences within a species.

Conclusion

Overall genetic diversity within each of the two genomes (L and R) is very low but not surprising, considering the northern location of these populations at the distribution edge. Due to its hybrid status that is restored every generation and the formation of polyploids within the population, *R. esculenta* does not suffer direct negative fitness effects and even seems to be expanding its range. So, these pure hybrid populations have managed to achieve independence from their parental species and, at the same time, do not seem to be an evolutionary dead end at all. Although the area is rather small, we found genetical spatial structuring within the area which was best explained by isolation by distance and could not be attributed to ecological influences. Together with the pattern of genetic diversity within populations, we conclude that the distribution used to be more restricted. Although the ploidy levels are not reproductively isolated, we found that the gene flow between the genotypes is not completely unrestricted.

Acknowledgements

Several people helped with fieldwork including H.-U. Reyer, D. Christiansen, E. Embrechts, J. Loman, T. Garner, R. Muheim, J. Secondi, S. Gödderz and M. Tobler. We thank S. Röthlisberger and T. Garner for their help with extracting DNA and microsatellite development and Ecogenics for help in microsatellite analysis of two loci. B. Baldetorp, M. Johansson and E. Niederer provided invaluable assistance with flow cytometry analysis. Also thanks to the group of R. Zinkernagel at the University Hospital Zürich who kindly provided us access to their FACSCalibur and K. Tschannen and K. Fink for their assistance. Many thanks also to L. Excoffier for help with the population genetic analysis. Comments by H.-U. Reyer, H. Hotz and P. Wandeler greatly improved the manuscript. This work was supported by Swiss NF grant No. 31-64004.00 to H.-U. Reyer. Catching permits were provided by Länsstyrelsen i Skåne Län (552-18591-02,-522-9286-03,-552-6571-04).

Author contributions

M.A. and C.J. contributed equally to this work. Both authors carried out all field- and lab work together. M.A. performed statistical analyses and wrote the paper. Both authors discussed the results and C.J. commented on the manuscript.

References

Amos, W., and A. Balmford. 2001. When does conservation genetics matter? Heredity **87**:257-265.

Beebee, T. J. C. 1996. Ecology and conservation of amphibians. Chapman & Hall, London.

Beebee, T. J. C. 2005. Conservation genetics of amphibians. Heredity **95**:423–427.

Beebee, T. J. C., and G. Rowe. 2000. Microsatellite analysis of natterjack toad *Bufo calamita* Laurenti populations: consequences of dispersal from Pleistocene refugium. Biological Journal of the Linnean Society **69**:367-381.

Beebee, T. J. C., and G. Rowe. 2004. An introduction to molecular ecology. Oxford University Press Inc.

Borkin, L. J., I. A. Caune, M. M. Pikulik, and T. M. Sokolova. 1986. Distribution and structure of the green frog complex in the USSR. Studies of Herpetology:675-678.

Borkin, L. J., A. V. Korshunov, G. A. Lada, S. N. Litvinchuck, J. M. Rosanov, D. A. Shabanov, and A. I. Zinenko. 2004. Mass occurrence of polyploid green frogs (*Rana esculenta* complex) in eastern Ukraine. Russian Journal of Herpetology **11**:203-222.

Bullini, L. 1994. Origin and evolution of animal hybrid species. Trends in Ecology and Evolution **9**:422-426.

Christiansen, D., K. Fog, B. V. Pedersen, and J. J. Boomsma. 2005. Reproduction and hybrid load in all-hybrid populations of *Rana esculenta* water frogs in Denmark. Evolution **59**:1348-1361.

Dannewitz, J., G. E. Maes, L. Johansson, H. Wickström, F. A. M. Volckaert, and T. Järvi. 2005. Panmixia in the European eel: a matter of time. Proceedings of the Royal Society of London Series B **272**:1129-1137.

Dawley, R. M. 1989. An introduction to unisexual vertebrates. Pages 1-18 *in* R. M. Dawley and J. P. Bogart, editors. Evolution and Ecology of Unisexual Vertebrates. New York State Museum, Albany New York, USA.

Dowling, T. E., and C. L. Secor. 1997. The role of hybridization and introgression in the diversification of animals. Annual Review of Ecology and Systematics **28**:593-619.

Dufresne, F., and P. D. N. Hebert. 1994. Hybridization and origins of polyploidy. Proceedings of the Royal Society of London Series B **258**:141-146.

Ebendal, T. 1979. Distribution, morphology and taxonomy of the Swedish green frogs (*Rana esculenta* complex). Mitteilungen aus dem Zoologischen Museum in Berlin **55**:143-152.

Eikhorst, R. 1984. Untersuchungen zur Verwandtschaft der Grünfrösche: Verbreitung, Struktur und Stabilität von reinen *Rana esculenta*- Populationen. PhD Thesis. University of Bremen, Bremen, Germany.

Eikhorst, R. 1987. Der Laich des Teichfrosches *Rana esculenta* Linnaeus, 1758 in einer reinen Bastardpopulation (Anura: Ranidae). Salamandra **23**:122-131.

Eikhorst, R. 1988. Die Verteilung von diploiden und triploiden Larven des Teichfrosches *Rana esculenta* in einer reinen Bastardpopulation (Anura, Ranidae). Salamandra **24**:59-68.

ESRI, and Environmental Systems Research Institute. 1992-2002. ArcView GIS 3.3.HCL Technologies Ltd., New Delhi, India. *in*.

Excoffier, L., P. Smouse, and J. Quattro. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics **131**:479-491.

Felsenstein, J. 1993. PHYLIP (Phylogeny inference package) version 3.6 alpha, distributed by the author. Department of Genetics, University of Washington, Seattle.

Frankham, R. 1996. Relationship of genetic variation to population size in wildlife. Conservation Biology **10**:1500-1508.

Frankham, R., J. D. Ballou, and D. A. Briscoe. 2004. A primer of conservation genetics. Cambridge University Press, Cambridge.

Garner, T. W. J., S. Angelone, and P. B. Pearman. 2003. Genetic depletion in Swiss populations of *Rana latastei*: conservation implications. Biological Conservation **114**:371-376.

Garner, T. W. J., B. Gautschi, S. Röthlisberger, and H.-U. Reyer. 2000. A set of CA repeat microsatellite markers derived from the pool frog, *Rana lessonae*. Molecular Ecology **9**:2173-2175.

Garner, T. W. J., P. B. Pearman, and S. Angelone. 2004. Genetic diversity across a vertebrate species' range: a test of the central-peripheral hypothesis. Molecular Ecology **13**:1047-1053.

Graf, J.-D., and M. Polls Pelaz. 1989. Evolutionary genetics of the *Rana esculenta* complex. Pages 289-302 *in* R. M. Dawley and J. P. Bogart, editors. Evolution and Ecology of Unisexual Vertebrates. New York State Museum, Albany, New York, USA.

Guex, G. D., H. Hotz, and R. D. Semlitsch. 2002. Deleterious alleles and differential viability in progeny of natural hemiclonal frogs. Evolution **56**:1036-1044.

Günther, R. 1975. Zum natürlichen Vorkommen und zur Morphologie triploider Teichfrösche, "*Rana esculenta"* ,L., in der DDR (Anura, Ranidae). Mitteilungen aus dem Zoologischen Museum in Berlin **51**:145-158.

Günther, R. 1983. Zur Populationsgenetik der mitteleuropäischen Wasserfrösche des *Rana esculenta*-Synkleptons (Anura, Ranidae). Zoologischer Anzeiger **211**:43-54.

Günther, R. 1991. Europäische Wasserfrösche (Anura, Ranidae) und biologisches Artkonzept. Mitteilungen aus dem Zoologischen Museum in Berlin **67**:39-53.

Günther, R., T. Uzzell, and L. Berger. 1979. Inheritance patterns in triploid *Rana "esculenta"* (Amphibia, Salientia). Mitteilungen aus dem Zoologischen Museum in Berlin **55**:35-57.

Hellriegel, B., and H.-U. Reyer. 2000. Factors influencing the composition of mixed populations of a hemiclonal hybrid and its sexual host. Journal of Evolutionary Biology **13**:906-918.

Hitchings, S. P., and T. J. C. Beebee. 1998. Loss of genetic diversity and fitness in common toad (*Bufo bufo*) populations isolated by inimical habitat. Journal of Evolutionary Biology **11**:269-283.

Holenweg Peter, A.-K. 1999. Dispersal and population dynamics in water frogs, *Rana lessonae*, *R. ridibunda* and their hybridogenetic associate *R. esculenta*. PhD Thesis. University of Zurich, Zurich, Switzerland.

Holenweg Peter, A.-K., H.-U. Reyer, and G. Abt-Tietje. 2002. Species and sex ratio differences in mixed populations of hybridogenetic water frogs: The influence of pond features. Ecoscience **9**:1-11.

Hotz, H., T. Uzzell, G. D. Guex, D. Alpers, R. D. Semlitsch, and P. Beerli. 2001. Microsatellites: a tool for evolutionary genetic studies of western Palearctic water frogs. Mitteilungen aus dem Museum für Naturkunde in Berlin, Zoologische Reihe **77**:43-50.

Jakob, C. 2007. Structure and dynamics of pure hybridogenetic water frog populations of *Rana esculenta* in Southern Sweden. PhD-Thesis. University of Zurich, Switzerland.

Lampert, K. P., D. K. Lamatsch, J. T. Epplen, and M. Schartl. 2005. Evidence for a monophyletic origin of triploid clones of the amazon molly, *Poecilia formosa*. Evolution **59**:881-889.

Lampert, K. P., S. A. Rand, U. G. Mueller, and M. J. Ryan. 2003. Fine-scale genetic pattern and evidence for sex-biased dispersal in the tungara frog, *Physalaemus pustulosus*. Molecular Ecology **12**:3325-3334.

Lande, R., and S. Shannon. 1996. The role of genetic variation in adaptation and population persistence in a changing environment. Evolution **50**:434-437.

Langella, O. 1999. Populations 1.2.28. www.cnrs-gif.fr/pge/bioinfo/populations/index.php.

Lantmäterieverket. 1996. Kartex 2.10. *in*. Sverige.

Lesbarrères, D., C. R. Primmer, A. Laurila, and J. Merilä. 2005. Environmental and population dependency of genetic variability-fitness correlations in *Rana temporaria*. Molecular Ecology **14**:311-323.

Masterson, J. 1994. Stomatal size in fossil plants: evidence for polyploidy in majority of angiosperms. Science **264**:421-423.

Meirmans, P. G., E. C. Vlot, J. C. M. Den Nijs, and S. B. J. Menken. 2003. Spatial ecological and genetic structure of a mixed population of sexual diploid and apomictic triploid dandelions. Journal of Evolutionary Biology **16**:343-352.

Menken, S. B. J., E. Smit, and J. C. M. Den Nijs. 1995. Genetical population structure in plants: Gene flow between diploid sexual and triploid asexual dandelions (*Taraxacum* section *ruderalia*). Evolution **49**:1108-1118.

Merilä, J., and A. J. Baker. 1996. Genetic population structure and gradual northward decline of genetic variability in the greenfinch (*Carduelis chloris*). Evolution **50**:2548-2557.

Michels, E., E. Audenaert, R. Ortells, and L. De Meester. 2003. Population genetic structure of three pond-inhabiting *Daphnia* species on a regional scale (Flanders, Belgium). Freshwater Biology **48**:1825-1839.

Muller, H. J. 1964. The relation of recombination to mutational advance. Mutation Research **1**:2-9.

Nei, M. 1987. Molecular evolutionary genetics. Columbia University Press, New York.

Otto, S. P., and J. Whitton. 2000. Polyploid incidence and evolution. Annual Review of Genetics **34**:401-437.

Pala, I., and M. M. Coelho. 2004. Contrasting views over a hybrid complex: between speciation and evolutionary "dead-end". Gene **347**:283-294.

Palo, J. U., D. S. Schmeller, A. Laurila, C. R. Primmer, S. L. Kuzmin, and J. Merilä. 2004. High degree of population subdivision in a widespread amphibian. Molecular Ecology **13**:2631-2644.

Plenet, S., P. Joly, F. Hervant, E. Fromont, and O. Grolet. 2005. Are hybridogenetic complexes structured by habitat in water frogs? Journal of Evolutionary Biology **18**:1575-1586.

Richards, A. J. 1973. The origin of *Taraxacum* agamospecies. Botanical Journal of Linnean Society **66**:189-211.

Rousset, F. 1997. Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. Genetics **145**:1219-1228.

Rowe, G., and T. J. C. Beebee. 2001. Fitness and microsatellite diversity estimates were not correlated in two outbred anuran populations. Heredity **87**:558-565.

Rowe, G., T. J. C. Beebee, and T. Burke. 1998. Phylogeography of the natterjack toad *Bufo calamita* in Britain: genetic differentiation of native and translocated populations. Molecular Ecology **7**:751-760.

Rowe, G., T. J. C. Beebee, and T. Burke. 1999. Microsatellite heterozygosity, fitness and demography in natterjack toads *Bufo calamita*. Animal Conservation **2**:85-92.

Rowe, G., T. J. C. Beebee, and T. Burke. 2000. A microsatellite analysis of natterjack toad, *Bufo calamita*, metapopulations. Oikos **88**:641-651.

Rybacki, M., and L. Berger. 2001. Types of water frog populations (*Rana esculenta* complex) in Poland. Mitteilungen aus dem Museum für Naturkunde in Berlin, Zoologische Reihe **77**:51-57.

SAS Institute. 2002-2003. Version 9.1.3 SP3 for Windows. SAS Institute Inc., Cary, NC.

Schneider, S., D. Roessli, and L. Excoffier. 2000. Arlequin: A software for population genetics data analysis. Ver 2.000. Genetics and Biometry Lab, Dept. of Anthropology, University of Geneva.

Schultz, R. J. 1969. Hybridization, unisexuality, and polyploidy in the teleost *Poeciliopsis* (Poeciliidae) and other vertebrates. American Naturalist **103**:605-619.

Seehausen, O. 2004. Hybridization and adaptive radiation. Trends in Ecology and Evolution **19**:198-207.

Seppä, P., and A. Laurila. 1999. Genetic structure of island populations of the anurans *Rana temporaria* and *Bufo bufo*. Heredity **82**:309-317.

Sjögren, P. 1991. Genetic variation in relation to demography of peripheral pool frog populations (*Rana lessonae*). Evolutionary Ecology **5**:248-271.

Slate, J., and J. M. Pemberton. 2002. Comparing molecular measures for detecting inbreeding depression. Journal of Evolutionary Biology **15**:20-31.

Soltis, D. E., and P. S. Soltis. 1999. Polyploidy: recurrent formation and genome evolution. Trends in Ecology and Evolution **14**:348-352.

Soltis, D. E., and P. S. Soltis. 2000. The role of genetic and genomic attributes in the success of polyploids. Proceedings of the National Academy of Sciences of the United States of America **97**:7051-7057.

Som, C., and H.-U. Reyer. 2006. Demography and evolution of pure hybridogenetic frog (*Rana esculenta*) populations. Evolutionary Ecology Research **8**:1235–1248.

Tallmon, D. A., W. C. Funk, W. W. Dunlap, and F. W. Allendorf. 2000. Genetic differentiation among long-toed salamander (*Ambystoma macrodactylum*) populations. Copeia **2000**:27-35.

Tegelström, H., and P. Sjögren-Gulve. 2004. Genetic differentiation among northern european pool frog (*Rana lessonae*) populations. Herpetological Journal **14**:187-193.

Tunner, H. G. 1974. Die klonale Struktur einer Wasserfroschpopulation. Zeitschrift für zoologische Systematik und Evolutionsforschung **12**:309-314.

Tunner, H. G. 1992. Locomotory behaviour in water frogs from Neusiedlersee (Austria, Hungary). 15 km migration of *Rana lessonae* and its hybridogenetic associate *Rana esculenta*. Pages 449-452 *in* Z. Korsos and I. Kiss, editors. Proc 6th Ord. Gen. Meeting S. E. H., Budapest 1991.

Uzzell, T., R. Günther, and L. Berger. 1977. *Rana ridibunda* and *Rana esculenta*: a leaky hybridogenetic system (Amphibia Salientia). Proceedings of the Academy of Natural Sciences of Philadelphia **128**:147-171.

Van Buskirk, J. 2005. Local and landscape influence on amphibian occurence and abundance. Ecology **86**:1936-1947.

Vinogradov, A. E., L. J. Borkin, R. Guenther, and J. M. Rosanov. 1990. Genome elimination in diploid and triploid *Rana esculenta* males: cytological evidence from DNA flow cytometry. Genome **33**:619-627.

Vorburger, C. 2001. Fixation of deleterious mutations in clonal lineages: evidence from hybridogenetic frogs. Evolution **55**:2319-2332.

Vrijenhoek, R. C. 1984. Ecological differentation among clones: The frozen niche variation model. Pages 217-231 *in* K. Wohrmann and V. Loeshcke, editors. Population Biology and Evolution. Springer-Verlang, Berlin-Heidelberg-New York.

Wright, S. 1978. Evolution and the genetics of populations. Volume 4. Variability within and among natural populaitons. University of Chicago Press, Chicago.

Zeisset, I., and T. J. C. Beebee. 2001. Determination of biogeographical range: an application of molecular phylogeography to the European pool frog *Rana lessonae*. Proceedings of the Royal Society Biological Sciences, Series B **268**:933-938.

Zeisset, I., G. Rowe, and T. J. C. Beebee. 2000. Polymerase chain reaction primers for microsatellite loci in the north European water frogs *Rana ridibunda* and *R. lessonae*. Molecular Ecology **9**:1173-1174.

Table 1. Gamete production in females and males for the three hybrid genotypes and offspring types arising from the nine potential mating combinations in a pure hybrid population of *R. esculenta*. Female LR can produce both diploid eggs and haploid eggs. Offspring with the parental genotypes (LL and RR) in the grey boxes are originally produced, but do not survive until reproductive status (Jakob 2007 & chapter 2).

Females \ Males	LR (R)	LLR (L)	LRR (R)
LR (LR) (R)	LRR / RR	LLR / LR	LRR / RR
LLR (L)	LR	LL	LR
LRR (R)	RR	LR	RR

Table 2. Investigated 33 ponds of *R. esculenta* in Scania (Southern Sweden).

Pond	Total sample size n=	Sampling year	Pond size (m^2)	Pond location	Canopy type
001	162	all	551	Core	Open
010	28	2002	5528	Core	Open
011	237	all	1316	Core	Open
012	28	2002	1436	Core	Open
014	186	all	1157	Core	Open
021	39	2002	2365	Core	Open
023	41	2002	4494	Core	Open
024	30	2002	918	Core	Open
032	205	all	5668	Core	Forest edge
032A	154	all	2278	Core	Forest
050	38	2004	836	Periphery	Forest edge
089	272	all	4887	Periphery	Open
101	42	2002	3610	Core	Forest
102	143	all	1889	Core	Forest
108	190	all	1242	Core	Forest edge
108A	37	2002	138	Core	Forest edge
111	173	all	3573	Core	Forest edge
112	36	2002	3093	Core	Open
123	19	2002	304	Core	Open
126	235	all	1115	Core	Forest edge
134	208	all	930	Core	Forest
135	34	2002	2287	Core	Open
137	24	2002	1439	Periphery	Forest edge
138	154	all	4791	Periphery	Forest edge
139	36	2003	2554	Periphery	Open
142	31	2004	836	Periphery	Open
147	31	2004	17286	Periphery	Forest edge
151	30	2004	1325	Periphery	Open
154	29	2004	1595	Periphery	Open
155	30	2004	2349	Periphery	Open
159	30	2004	1367	Periphery	Open
160	30	2004	12035	Periphery	Forest edge
161	30	2004	1881	Periphery	Forest

Sampling year: all: ponds were sampled from 2002 to 2004. Pond numbers and location according to the map (Fig. 1); core ponds are situated within a radius of 6 km of the centre; peripheral populations are located more at the border of the distribution. Pond type according to ecological classification: open = no shaded shore line, forest edge = partly shaded due to some trees, forest = pond fully surrounded by trees.

Table 3. Polymorphic microsatellite loci used in this study. Loci Ca18 and Ca5 were species-specific for the L-genome; Re2CAGA3 was species-specific for the R-genome and the other four loci amplified in both genomes.

Locus	Electrophoresis system	L-alleles	R-alleles
Ca18	Elchrom SEA 2000	183/189	-
Ca5	Elchrom SEA 2000	258/262/266	-
Re2CAGA3	Elchrom SEA 2000	-	170/198/202/210/214/218/222
Ca1b5	Elchrom SEA 2000	121	135/137
Re1CAGA10	Elchrom SEA 2000	92/98	96/108/110/114/120/124
Ca1b6	ABI Prism3100	79	86/93/98
Ga1a19	ABI Prism3100	197	201/203/207

Table 4. Nei genetic diversity (GD) (Nei 1987) calculated for the L- and the R-genome in 33 ponds in Southern Sweden. Diversity is based on 3 polymorphic microsatellite loci for L-genomes and on 5 polymorphic microsatellite loci for R-genomes. Also shown are means over all ponds with standard errors.

Pond	GD in L	GD in R
001	0.259	0.409
010	0.315	0.640
011	0.273	0.713
012	0.440	0.656
014	0.354	0.810
021	0.436	0.843
023	0.440	0.815
024	0.391	0.903
032	0.466	0.951
032A	0.546	0.956
050	0.460	0.817
089	0.160	0.351
101	0.398	0.883
102	0.379	0.896
108	0.603	0.876
108A	0.534	0.907
111	0.345	0.953
112	0.412	0.975
123	0.306	0.597
126	0.529	0.945
134	0.384	0.788
135	0.373	0.626
137	0.000	0.708
138	0.469	0.163
139	0.304	0.856
142	0.345	0.881
147	0.170	0.806
151	0.548	0.648
154	0.000	0.726
155	0.417	0.048
159	0.508	0.051
160	0.047	0.296
161	0.502	0.299
Mean	**0.364 (0.028)**	**0.691 (0.047)**

Table 5. Variance components from analysis of molecular variance (AMOVA) for the two genomes (L and R) between sexes (female/male), genotypes (LR/LLR/LRR), years and among populations. Negative variance components usually indicate an absence of genetic structure, although in some cases they can have a biological meaning (Excoffier, http://anthro.unige.ch/arlequin).

Genome	Source of variation	df	Sum of squares	% variation	*P*-value
L	Among years	2	0.533	-0.33	0.951
R	Among years	2	2.750	-1.20	0.990
L	Among sexes	1	1.245	0.19	0.054
R	Among sexes	1	0.861	-0.20	0.877
L	Among genotypes	2	2.432	0.36	**0.001**
	LRR – LR/LLR				**<0.001**
	LLR – LR/LRR				0.674
	LR – LRR/LLR				0.345
R	Among genotypes	2	19.361	1.05	**<0.001**
	LRR – LR/LLR				0.319
	LLR – LR/LRR				0.662
	LR – LRR/LLR				**<0.001**
L	Among populations	32	36.703	5.86	**<0.001**
R	Among populations	32	310.283	18.53	**<0.001**

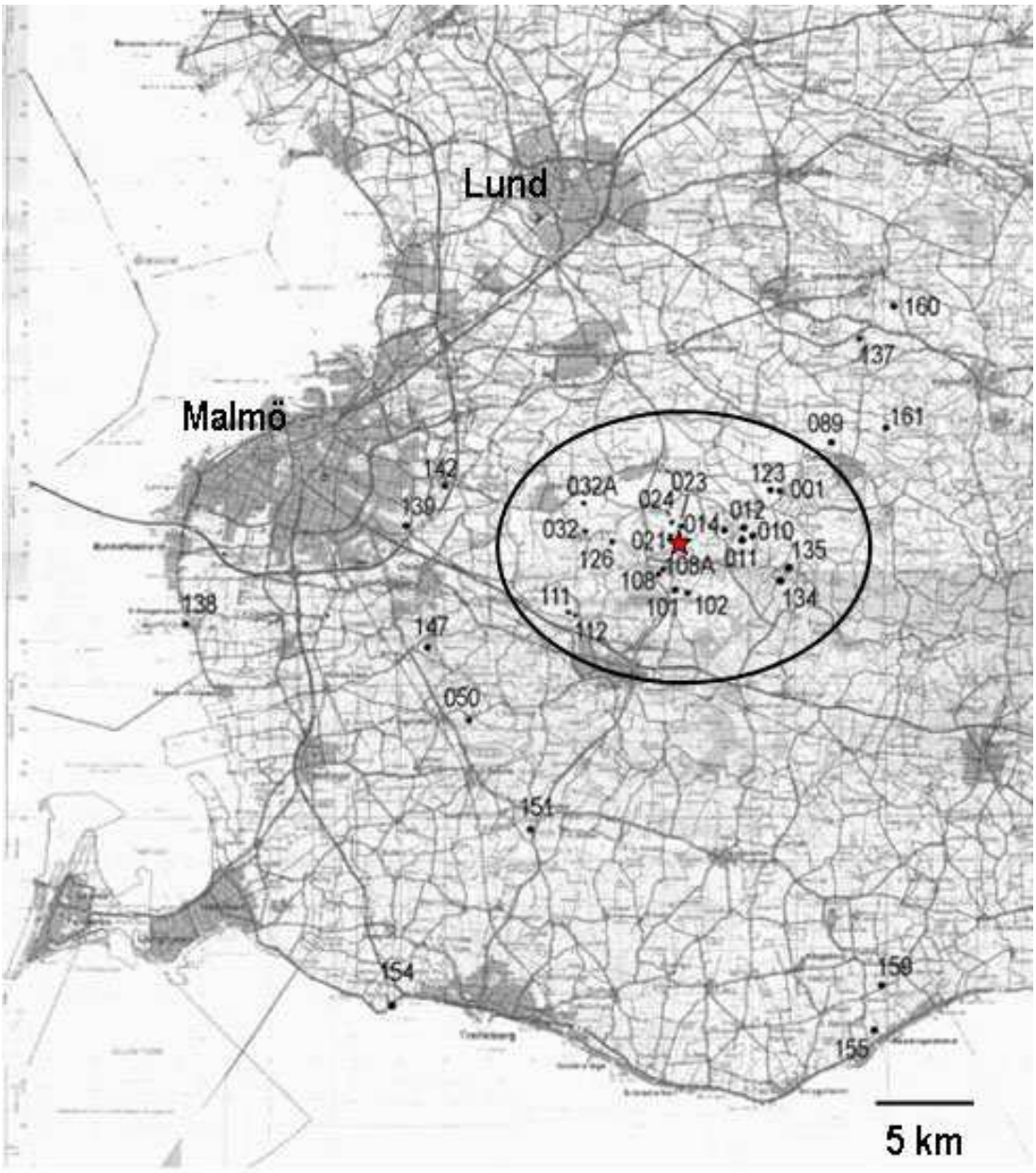

Figure 1. Distribution of the 33 sampling sites in Southern Sweden (Scania). Sampled in all years: 001, 011, 014, 032, 032A, 089, 102, 108, 111, 126, 134 and 138. Sampled only in 2002: 010, 012, 021, 023, 024, 101, 108A, 112, 123, 135 and 137. In 2003 we sampled pond 139 and in 2004: 050, 142, 147, 151, 154, 155, 159, 160 and 161. Ponds within the ellipse are considered core ponds. Star indicates the centre of the distribution from which geographic location within the area was calculated.

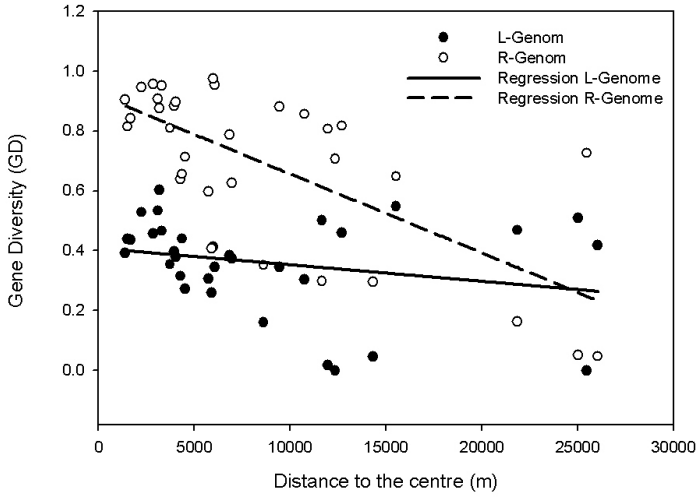

Figure 2. Relationship between gene diversity (Nei 1987) and geographic location of ponds within the distribution in Southern Sweden. Pond location is expressed by its distance to the centre of the study area (see Fig. 1).

a) b)

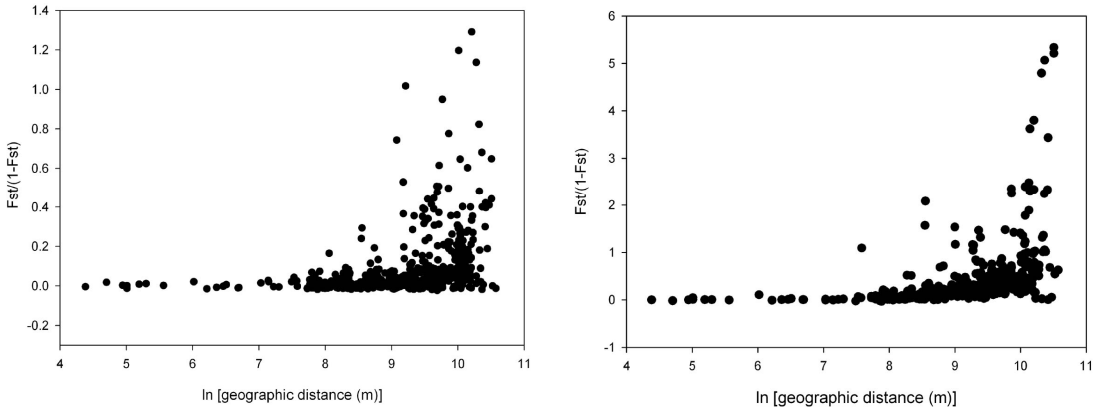

Figure 3. Isolation by distance in *R. esculenta* showing the pairwise genetic distances as a function of corresponding pairwise spatial (ln) distances following Rousset (1997) for the two genomes separately: a) L-genome b) R-genome (Mantel test with 10’000 permutations).

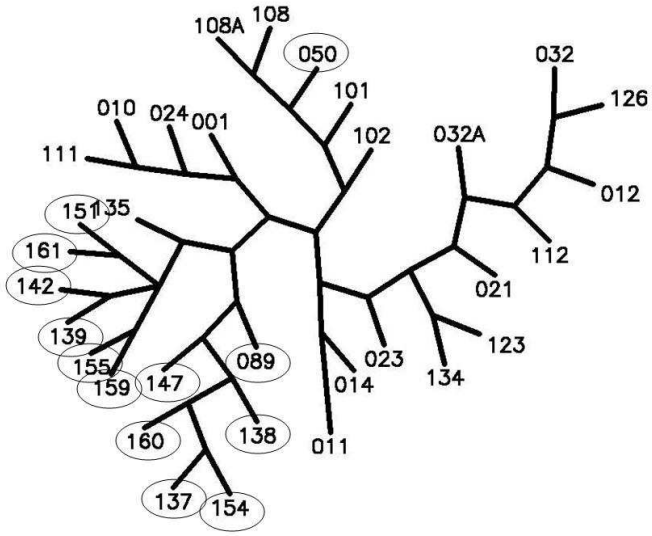

Figure 4. Consensus tree from the L- and R-genomes calculated from Cavalli-Sforza distances (Neighbour-Joining method) between the 33 populations. Encircled are the populations situated at the periphery.

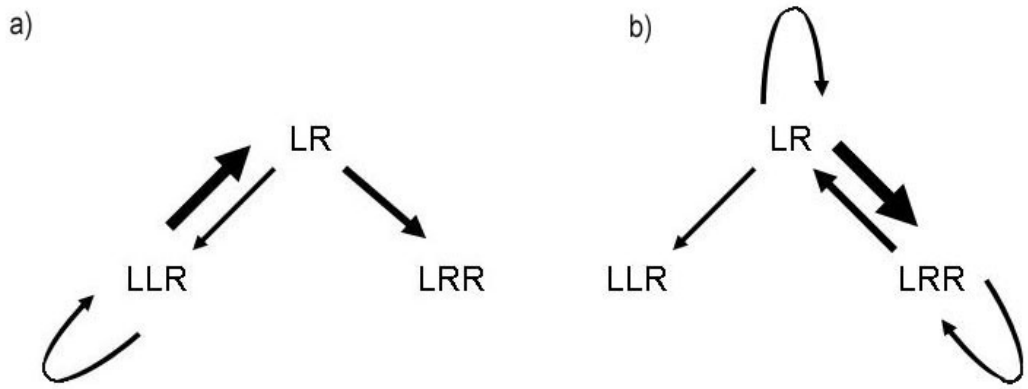

Figure 5. Inheritance pathways of a) an L-allele b) an R-allele in populations consisting of diploid (LR) and triploid (LLR, LRR) *R. esculenta* individuals. Width of the arrows indicates how often an allele travels from one genotype to another, based on the assumption of random mating and Table 1.

Appendix 1.

Locus	Repeat motif	Primer sequences (5'-3')	Ta (℃)	Size (bp)	GenBank Accession no.
Ga1a19	$(CT)_{27}$	F: GACTGGGAGGGATAGGAAGG R: CAGGGGATTTTCCCATCAG	53℃	140	EF121547
Ca1b6	$(TG)_{13}$	F:AAACTCGCGGTTTCCCTTAG R:GAGCCAGGTTAAGATAACTGGAG	59℃	90	EF121548
Re1CAGA10	$(TG)_9GC(TG)_{13}TA(TC)_3$	F:CATGTTTACCGTCACTTTAAGAACAC R:CATCTCTTCAGGTGGCTGGA	58℃	108	EF121549
Re2CAGA3	$(CAGA)_6TTA(GATA)_1(GACA)_1$ $(GATA)_{20}(GACA)_1$	F: ATGTCGTTAGAGTTCATAGG R: ATCTCAAGTAATCTGTCTGTC	60℃†	214	EF121550

† Initial annealing temperature in touchdown protocol (see text)

CHAPTER 5

Origin of the pure hybrid populations of *Rana esculenta* in Southern Sweden – a phylogenetic approach

MARTINA ARIOLI & CHRISTIAN JAKOB

Abstract.-Colonization patterns of species and their recent distribution can explain much of the genetic variation within and between populations and their evolutionary perspective. The edible frog *R. esculenta* is a water frog hybrid taxon arising from the mating between *R. ridibunda* and *R. lessonae*. Its distribution is rather well known, it covers most of Central Europe south of 44 degrees of latitude and ranges into Russia. Large parts of the distribution are shared with at least one of the parental species, except in the north where areas with pure hybrid populations are found. In order to learn more about the all-hybrid populations in Sweden, which are nowadays isolated from Central Europe, we investigated water frog populations all around the Baltic Sea. Using microsatellite markers and mitochondrial DNA sequences we examined their population composition, genetic diversity and the genetic relationships between them.

All-hybrid populations were found in Southern Sweden, in Denmark, on the northern coast of Germany and in Poland. In the other areas the genotypic population composition varied strongly. Genetic diversity was decreasing the further north and west a population was located within the investigated area. Such a decreased genetic diversity in northern populations is not unusual and most likely explained by a post-glacial recolonization from a southern refugium. However, with neither of the genetic markers (microsatellite and mtDNA) could we identify an exact colonization route into Fennoscandia for the water frog taxon *R. esculenta*. It is known that the topography looked quite different at the time when postglacial migration of these frogs reached this northern area (approximately 9500 years BP): Southern Sweden, Denmark and Northern Germany were all still connected by dry land. The poor separation of these populations around the Baltic Sea into clear clusters indicates that these frogs were then able to migrate between these areas more easily that we would expect nowadays based on current sea barriers. Nevertheless, the occurrence of endemic mtDNA haplotypes in Central and Southern Sweden suggests that these populations have been isolated from the rest for a certain time.

Introduction

The Pleistocene (1.8 myr – 11.5 kyr ago) with its recurring ice ages influenced the European ecosystems strongly (Andersen and Borns 1997, Hewitt 2000). The ice ages had a periodicity of about 100 kyr with relatively short warm interglacial periods. During the cold periods the glaciers were advancing rapidly and most parts of Europe were covered with ice. These expanding ice sheets forced species to retract into areas further south which were ice-free. Several such refuge areas have been identified and described (Hewitt 1999, 2000, Petit et al. 2003): the peninsulas of Iberia and Italy, the Balkans and Greece. During the warmer interglacial periods, species surviving in these refugia started recolonizing the newly available areas which previously had been glaciated. Since much of the water was still bound in the massive ice sheets, the sea level was distinctively lower and land bridges, such as a connection between Britain and Continental Europe, were present (Lambeck 1995, Snell et al. 2005). Additionally, when the glacial period ended, the southern part of the Baltic area was freed from the heavy pressure of the ice sheet and subjected to a very rapid rising of the land (Gislén and Kauri 1959).

The southern refugia have contributed very differently to the post-glacial colonization of Europe. Since the Alps and the Pyrenees formed severe barriers to the colonization routes from Italy and Spain (Hewitt 1996), most species dispersed into Europe from the Balkan refugium (Taberlet et al. 1998, Hewitt 1999), but the phylogeographical patterns vary substantially between species. Some species dispersed from just one refugium, others colonized from several refugia, and the different lineages met somewhere in Europe, consequently building hybrid zones (so-called “suture zones”). In Europe, four such suture zones are recognized; the Alpine barrier, the Pyrenees, one zone in central Europe where lineages from the Iberic refugium meet lineages from the eastern regions, and one zone in the middle of Scandinavia. This Scandinavian conjunction has been shown for several mammalian species (e.g. *Sorex araneus*, *Clethrionomys glareolus*, *Microtus agrestis* and *Ursus arctos*) and might be the reason why their genetic diversity seem to be higher than expected (reviewed by Taberlet et al. 1998, Petit et al. 2003). Only recently have amphibians become the focus of such phylogenetic studies in Europe (Wallis and Arntzen 1989, Zeisset and Beebee 2001, Babik et al. 2004, Palo et al. 2004, Snell et al. 2005).

The water frog *Rana esculenta* is a hybrid taxon resulting from the mating between the pool frog, *Rana lessonae* and the lake frog, *Rana ridibunda*. Usually hybrid taxa are limited to narrow overlap zones between the two hybridizing species

and maintained through spatial or ecological processes, which are described by the tension zone model (Barton and Hewitt 1985) or the mosaic model (Harrison and Rand 1989). In some cases, however, hybrids have reached large distributions and are ecologically and evolutionarily very successful. Among them are fishes (Quattro et al. 1992, Vrijenhoek 1994, Alves et al. 2001), salamanders (Hedges et al. 1992) and also the water frog taxon *R. esculenta*. The water frog was first described by Linné in 1758 and regarded as a true species up to the late 1960s when Berger (1967) discovered the hybrid character of this taxon through crossing experiments. The geographical distribution of *R. esculenta* ranges over a large part of Central Europe into Eastern Europe and overlaps with the distribution range of the two parental species (*R. lessonae* or *R. ridibunda*) (Fig. 1).

Due to its special mode of reproduction, namely hybridogenesis (Schultz 1969), *R. esculenta* is forced into coexistence with one of the parental forms. However, the described populations compositions are manifold and differ depending on the geographical region (Günther 1974, Plötner 2005). Given the reproductive dependency of the hybrid on the parental species it is rather surprising that in some areas, especially in the most northern part of the distribution, pure hybrid populations have been established (Ebendal 1979, Fog 1994). These all-hybrid populations consist of the normal diploid hybrid (LR) and triploid forms (LLR and/or LRR).

The northern distribution border of *R. esculenta* includes all of Denmark and runs through Sweden and Estonia. Regarding the Swedish distribution, Gislén and Kauri (1959) reported a common occurrence of *R. esculenta* in Western Scania (Southern Sweden) and sporadic populations along the east coast up to the latitude of Stockholm. The authors assumed that the distribution in Sweden must have been much larger and continuous in earlier days, but due to today's more severe climate, most of these populations along the east coast have become extinct. Since then, this pattern of extinction has even advanced, and some of the populations described by Gislén and Kauri no longer exist (J. Pröjts personal communication). Additionally, Ebendal (1979) reported several locations in Eastern Scania, which apparently had *ridibunda* like frogs, but recent investigations could no longer detect water frogs in these populations (Kvindal 1998, J.Pröjts pers. comm.). However, through intensive search we were able to discover one presumably viable, but rather isolated population in Östergötland (Jakob 2007 & chapter 2).

The dramatic changes in the Swedish distribution of *R. esculenta* give the area in Southern Sweden a special status. First, this location lies at the border of the distribution area, and second the area is isolated due to contemporary sea barriers in the south and unsuitable conditions or lacking habitat in the north. Both these facts

could have consequences on the viability and the long-term evolution of these populations.

It has recently been shown that Fennoscandia has been colonized by another *Rana* species, *Rana temporaria*, about 10000 years ago and that postglacial recolonization into Fennoscandia proceeded via two routes: from the south through Denmark and from the southeast through Finland and Northern Sweden. This resulted in a suture zone in middle Sweden (Palo et al. 2004). By intensive sampling of the area around the Baltic Sea, we investigated possible colonization scenarios by *R. esculenta* into Sweden. Additionally, we focussed on the "remaining" isolated population in Östergötland. It is known from Britain that *R. lessonae* and *R. ridibunda* have been introduced (Zeisset and Beebee 2001, 2003), e.g., for food reasons, and subsequently established viable populations. For Sweden introductions of French frogs have been described as well, but these populations are thought to be extinct nowadays (Söderbäck 1984). We therefore wanted to investigate if these frogs in Östergötland are truly native - and thus worth protecting - or if they are introduced from another country.

We asked the following questions with regard to the sample populations around the Baltic Sea: (1) What population compositions are found around the Baltic Sea and is composition related to genetic diversity and geographic location? (2) How are the Southern Swedish populations related those populations around the Baltic Sea, and can we draw any conclusions about the origin of these Swedish populations and possible colonization scenarios? (3) Is the isolated population in Östergötland introduced or native to this area?

We approached these questions by examining genetic variation in mitochondrial DNA (ND2 & ND3 gene) and microsatellite markers in *R. esculenta* populations covering the geographic region around the Baltic Sea.

Methods

Samples and genotype determination

In total, 1526 water frogs from 52 different populations were sampled for the analyses. Twelve of these populations are situated in Sweden, nine in Denmark, fourteen in Germany, eight in Poland, and three each in Estonia, Latvia and Lithuania (Table 1). Most individuals were collected as adults or juveniles, only those from the population near Uppsala (Sweden) were sampled as metamorphs.

The classification into taxa (*R. esculenta*, *R. lessonae* or *R. ridibunda*) and within the hybrid into genotypes (either diploid (LR) or triploid hybrid (LLR, LRR)) was done by microsatellite analyses and, if blood was available (only for samples from Southern and Central Sweden), also with flow cytometry (according to Jakob 2007). Most individuals could be assigned unambiguously to one of the genotypes with these methods. In some cases, however, results from different methods and/or microsatellite markers were not corresponding, despite repeated analysis. These individuals were categorized separately and named “mixed” individuals.

Molecular genetic methods

Genetic variation was assessed with two mtDNA gene sequences (ND2 & ND3) and at eight nuclear microsatellite loci. The template DNA was extracted using QIAamp® DNA mini kit (Qiagen) or BioSprint^{TM} (Qiagen) from approximately half a toe clip per sampled individual.

Sequencing of the ND3 gene (340 bp) was done for 140 individuals and additional sequences of ND2 (1038 bp) for 52 individuals were obtained from 32 different populations. Double stranded amplifications and cycle sequencing were carried out according to the protocol listed in Table 2. Amplified DNA was purified using QIAquick PCR purification kits (QIAGEN) after a control separation of 8 µl of the reaction product in 1.4% agarose gels. A Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) was used for the sequencing reaction. Sequencing analysis (electrophoresis and detection of the fluorescent dye labeled nucleotide fragments) was performed with an automatic DNA sequencer (ABI Prism 377). Sequences were aligned using the CLUSTAL W algorithm (Higgins and Sharp 1989) and proof-read by visual inspection.

When only considering the short fragment (ND3) the variability was very low; we detected only six different haplotypes two of which were ridibunda-specific and four were lessonae-specific. When considering both genes together, the variability was higher and, therefore, the resolution was much better. Consequently, the analysis

was based on the 52 individuals for which we had the whole ND2&ND3 sequence (1378 bp). In addition to the samples listed in Table 1, we included samples from Germany (2 LL, 1 RR, 1 LR), Italy (2 LL), Romania (2 LL), Poland (1 LL) and Slovakia (1 RR) which were kindly provided by J. Plötner, F. Köhler, K.-J. Schultze, T. Ohst, H. Hotz and G. Guex (Table 3).

Microsatellite analysis was performed on all 1526 individuals from all 52 locations using the following 8 microsatellite primers: Ca1b5, Ca5, Ca18 (Garner et al. 2000), Res16 (Zeisset et al. 2000) and Ca1b6, Re1CAGA10, Re2CAGA3, Ga1a19 (chapter 4). PCR amplifications were performed in a total reaction volume of 10 µl, under conditions described in Jakob et al. (2007); allele scoring was also conducted as specified in this paper.

Data analysis

The frequency of L- resp. R-genome per population was calculated as the number of L- resp. R-genome per population divided by the total number of genomes in this population. Geographical trends in proportion of L- resp- R-genome were examined with linear regression analyses using SAS 9.1.3 SP3 for Windows (SAS Institute 2002-2003).

Phylogenetic analyses for the mtDNA were carried out on the basis of the combined sequences of the ND2 and ND3 gene (1378 bp). The partition-homogeneity test (Farris et al. 1994) implemented in PAUP*4.0b10 (Swofford 2002) was used to test whether the two datasets could be combined (ND2 versus ND3). A haplotype network was generated using the statistical software ARLEQUIN (Schneider et al. 2000), which constructs the most probable minimum-spanning network using pairwise distances. Tree constructions were made with distance matrix methods (neighbour-joining NJ) and maximum parsimony method (MP) using the program MEGA version 3.1 (Kumar et al. 2004). The NJ analysis was based on the model of Tamura-Nei (Tamura and Nei 1993). The most parsimonious (MP)-tree was searched using Close-neighbour-interchange (CNI = 1). Tree robustness was evaluated by bootstrapping (Felsenstein 1985) for both neighbour-joining and parsimony analysis with 1000 replicates.

Additionally, for the phylogenetic analyses we included heuristic searches under maximum likelihood criterion (ML) using PAUP*4.0b10 (Swofford 2002). Confidence values for the nodes were obtained by bootstrapping (100 replicates). ForCon (Raes and Van de Peer 1999), a software tool for the conversion of sequence alignments, was further applied. The best evolutionary model of nucleotide substitution that fit the data was obtained by using the hierarchical likelihood ratio test as implemented in

Modeltest 3.5 (Posada and Crandall 1998). The best fitting model proved to be TrN+G (TrN: Tamura-Nei model (Tamura and Nei 1993), G: γ correction). The likelihood-estimated substitution rates were $R_{(A-C)}$ = 1.0000, $R_{(A-G)}$ = 24.8627, $R_{(A-T)}$ = 1.0000, $R_{(C-G)}$ = 1.0000, $R_{(C-T)}$ = 3.3095, and $R_{(G-T)}$ = 1.0000. The base frequencies were estimated at 0.2512 (A), 0.3065 (C), 0.1215 (G), and 0.3209 (T). The rate heterogeneity among variable sites was estimated to follow a gamma distribution with the shape parameter α = 0.0160. This model of nucleotide substitution (TrN+G) with the corresponding settings was used for the ML analyses of the combined (ND2 + ND3) data set.

Population genetics analyses based on microsatellite data were performed using PHYLIP 3.6 (Felsenstein 1993) and ARLEQUIN (Schneider et al. 2000). Since there is no recombination between L- and R-genomes, the analyses were done separately for each genome. Allele frequencies for each of the 52 populations were determined. We calculated genetic diversity (Nei 1987) for each population. Geographical trends in genetic diversity and average of allele number were examined with a multiple linear regression analysis using SAS 9.1.3 SP3 for Windows (SAS Institute 2002-2003). To examine relationships between populations we constructed unrooted neighbour-joining (NJ) trees (based on Nei's distances) and the reliability of the tree was evaluated by bootstrapping (1000 replicates).

Results

Population composition

We found a wide variety of population compositions in our sample (Table 4, Fig. 2). Pure *R. lessonae* populations were only found near Uppsala and in Estonia, the most northern populations in our study. The "classical" population composition, consisting of the hybrid (LR) and one of the parental species (either LL or RR), was present in the Northeast, mainly in the Baltic states, Poland and in Sweden (Östergötland). Twenty-nine of the investigated populations were pure hybrid populations, consisting of diploid individuals (LR) and either both or just one triploid type (LLR/LRR). With one exception, all populations in Southern Sweden were pure hybrid populations. This was also true for the German populations along the northern coast and for most Danish populations, except for the four populations on the island of Bornholm. Populations consisting of both hybrids (diploid and triploids) and parental individuals were found in Poland, on Bornholm and in two german populations situated further

inland (Rothemühl). For the description of the population systems we omitted the mixed animals in order to simplify the pattern. Although they were not present in all populations, these mixed individuals occurred in many populations at low frequency and were not specific to sites or countries. Tetraploid animals or triploid parental genotypes that were detected through flow cytometry in the Swedish samples could not be detected for this European comparison for which we only performed microsatellite analysis on toe clips (see Jakob 2007). Therefore, these unusual genotypes are not mentioned any further. The position of the populations (latitude and longitude) was correlated (Pearsons correlation coefficient = 0.489, $P < 0.001$), i.e. populations more east were also located more to the north (Fig. 2). Simple linear regression showed that the proportion of L-genomes in a population significantly increased with latitude as well as longitude (both $P \leq 0.003$, $R^2 \geq 0.159$). For the R-genome, the pattern was correspondingly reversed.

Microsatellite diversity

Out of the eight microsatellite loci (of which 5 amplify both the L- and R-genome), five were polymorphic for the L-genome and six were polymorphic for the R-genome. This resulted in an average of 3.7 alleles per locus for the L-genome and an average of 9.8 alleles per locus for the R-genome (Table 5). The intra-population diversity ranged from 0 (all loci were fixed) to 0.342 (L-genome) and 0.620 (R-genome), respectively. Across the region of Northern Europe, genetic diversity in the R-genome was mainly influenced by latitude when tested in linear regressions ($R^2 = 0.399$, $P < 0.001$). Populations towards the north seem to be genetically impoverished (Fig. 3a). For the L-genome we found that it is mainly longitude that influences intra-population genetic diversity ($R^2 = 0.302$, $P < 0.001$); diversity increased towards east (Fig. 3b). The same picture holds true for the average number of alleles per locus and population (data not shown). However, due to the position of our study populations it has to be noted that latitude and longitude are correlated (see above) and therefore not completely separable. The supposedly isolated population in Östergötland had, as expected, a low genetic diversity. In the R-genome this population was fixed in all loci and in the L-genome it had one locus that showed two alleles. None of the detected alleles in Östergötland were population-specific, they all occurred also in other populations in our sample. The most northern population sampled on the continent, a lessonae population from Hara (Estonia), was fixed in all loci.

Mitochondrial population differentiation

The partition homogeneity test indicated that the ND2 and ND3 gene partitions were not significantly mutually incongruent (P = 0.744) which justified the combination of both data sets. Out of the 62 individuals that were sequenced for the ND2 and ND3 gene, 59 frogs had *lessonae* mtDNA, whereas three frogs showed *ridibunda* mtDNA. Two of these exceptional frogs were from Latvia, one was from Lithuania. Since our focus is on the Swedish populations and none of these Swedish individuals possessed the *ridibunda* mtDNA type, we will not consider these three individuals any further but focus on the animals that had a *lessonae* mtDNA type. Within the *lessonae* mtDNA 24 variable sites (1.7% of the total sequence) defining 18 different haplotypes were observed among the 59 individuals (Fig. 4). When considering only the nonsynonymous substitutions that lead to a replacement in amino acids, we obtained 11 different haplotypes. Haplotypes differed between 1 and 12 base pairs. Table 6 lists the frequency and occurrence of haplotypes in the different populations.

The results of the NJ and MP methods for the phylogenetic tree were very similar (Fig. 5a). Several groups were clustering, although not very distinctly when considering the rather low bootstrap values. One branch was formed by individuals, originating from six different populations in Southern Sweden which all possessed a separate haplotype (1d, 2bp difference). Another branch was composed of frogs from the Östergötland population that all have the same haplotype (4) which differs from the main haplotype present in Southern Sweden and Uppsala (1a) by one base pair. The Italian *R. lessonae* (Nr.1 in Table 3), the *R. lessonae* from Teschendorf (Nr.5) and Mühlenbeck (Nr.6) and the two frogs from Oder River (Nr.7) were each forming separate branches. Additionally, there were two individuals from Hanstorf and two Polish individuals that clustered separately. The rest of the individuals which included frogs from Sweden, Denmark, Germany, Poland and the Baltic states were not separated. For the ML method the calculated phylogenetic tree looked slightly different (Fig. 5b). Here, only two groups were distinctively clustering; the two frogs from Teschendorf and Mühlenbeck as well as one individual each from Romania, Slovakia, Poland, Lithuania and Germany.

Microsatellite population differentiation

Similar to the mtDNA phylogenetic tree, the clustering of the populations based on microsatellite data was not very distinct either (Figs. 6 and 7). Again bootstrap values were somewhat low (mostly < 50%), indicating that the tree topography is not very reliable. For the L-genome basically three clades can be distinguished; one containing central Polish and Baltic populations, the second consisting of Danish

populations from Bornholm together with two peripheral Polish populations and the one near Uppsala and in the third cluster we find all the Swedish populations together with the coast populations of Germany and Poland as well as the remaining Danish populations. For the R-genome, we found a similar phylogenetic tree, although only consisting of more or less two clades; one was containing the Swedish populations and some of the German coast populations (also with one from Priedes, Latvia) and the other clade comprising the rest of the populations.

Discussion

Population composition

The population compositions we have found in the area around the Baltic Sea did not come as a surprise. The occurrence of pure hybrid populations along the northern coast of Germany has been described earlier (Günther 1974, Günther and Plötner 1990, Günther 1991), although - due to methodical problems - it was often not clear whether both triploid forms occur and in what relative frequencies. Our data show that the proportion of LLR is usually higher than the proportion of LRR; nevertheless did we find some LRR individuals in almost all these populations. The proportion of LRR individuals increases with latitude from Germany through Denmark and finally to Southern Sweden where the two triploid forms are about equally common (Jakob 2007 & chapter 2). The furthest east we have found triploid *R. esculenta* individuals was in Poland, although triploid individuals have been described as far as in Russia, Belarus and Ukraine (Okulova et al. 1997, Borkin et al. 2004). Despite this increase of LRR triploid towards the north, the relative frequency of R-genomes in a population decreases the further north and east a population lies, which is mainly due to the fact that no *R. ridibunda* frogs are found there. The two most northern populations that we sampled even were pure *R. lessonae* populations. In general, the frequency of L-genomes increases towards the northeast and consequently the proportion of R-genomes in a population decreases. It has been proposed earlier that *R. lessonae* (and therefore the L-genome) is better adapted to cooler and more humid areas and, therefore, is better suited to colonize northern regions (Günther 1990). This might also be linked to the fact that the two species, *R. lessonae* and *R. ridibunda*, have different habitat preferences and hibernation strategies. Whereas *R. lessonae* prefers smaller ponds with dense vegetation and hibernates on land, *R. ridibunda* is more often found in bigger ponds with little or no vegetation or stream habitats (Holenweg

Peter et al. 2002). *R. ridibunda* usually hibernates in water and is therefore very susceptible to hypoxic conditions if smaller ponds are covered with ice during winter. For Eastern Germany it has been suggested that it is this physiological restraint together with climatic conditions that explains the distribution pattern (Plötner 2005).

In Sweden, our main study area, we found three different populations systems, one in each of the three locations. As described earlier, the population near Uppsala is a pure *R. lessonae* population (Sjögren 1988) whereas pure hybrid populations are found in Southern Sweden (Jakob 2007 & chapter 2). Interestingly, the population in Östergötland turned out to be a combination between these two: a mixed *lessonae-esculenta* population system, but with no triploid forms. It is difficult to determine if one population system emerged from the other. We know from Southern Sweden that selection acts against the parental forms (LL and RR). It may well be that at higher latitudes *R. esculenta* comes increasingly under selection pressure that eliminates the triploid forms LLR and LRR earlier (i.e. in Central Sweden) than the diploid LR (Uppsala). But we lack experimental and field data on the larval development from these two northern areas, and hence, presently can not test this hypothesis. Interestingly, the same population system pattern with pure *R. lessonae* populations furthest north followed by *lessonae-esculenta* systems, without triploid hybrids at slightly lower latitudes is also predominant in the Baltic states. This suggests that these population systems are best adapted to high latitudes.

In Skåne it was rather surprising that in one pond (Skåne 154) we detected 3 adult *R. ridibunda* females. According to previous studies, the parental genotypes (LL and RR) die during the larval development so that none of these genotypes are left among the adults (Jakob 2007 & chapters 2 and 3). It could have dramatic effects on the population systems if *R. ridibunda* frogs establish themselves as breeders. The three individuals in pond 154 might have been introduced, but considering the mtDNA and microsatellite analyses (see below), this seems not very plausible. So far, we do not exactly know what imposes the selection on the parental genotypes during the larval period. Conditions in pond 154 might, at some point, have been so favourable that a few *R. ridibunda* survived to adulthood, but this needs definitely further investigations. In Swiss populations with different *R. esculenta* hemiclones in the same pond, Hotz et al. (1992) and Vorburger (2001) found the formation of *R. ridibunda* females from inter-hybrid matings. However, since apparently only females were formed, the authors concluded that independently reproducing populations of *R. ridibunda* can not arise in this way.

In Poland where many of the earlier studies on water frogs have been carried out (Berger and Berger 1992, Rybacki and Berger 1994, 2001), the population

systems are manifold. Practically all genotypes and population systems, including the ones we have detected in Sweden, are present in Poland, which is supported and explained by the differing hybridogenesis patterns found in this region (Berger 1983).

Genetic diversity

It is expected that the lowest genetic diversity is found in front of a colonization route and that diversity is highest near the refugium (Sage and Wolff 1986, Hewitt 1996, Ibrahim et al. 1996, Taberlet et al. 1998). This agrees with our results very well. When combining both genomes we find that diversity is highest in the south east (Poland, Central Germany) of the sampled area, corresponding to a region where presumably the colonization came from (Zeisset and Beebee 2001). The lowest diversity was found in the Southern Swedish populations as well as in the Danish populations. This is consistent with data from *R. temporaria* (Palo et al. 2004), where some Danish island populations had the lowest genetic diversity. Similarly, Snell et al. (2005) found that Swedish and Norwegian populations of *R. lessonae* were fixed for all alleles in the investigated microsatellite primers, although it has to be noted that sample sizes in this study were very low. In our investigation the number of sampled individuals per population was high (especially for the populations in Southern Sweden); therefore, we are confident that low genetic diversity in the northern region is a reality. In a recent study, Johansson et al. (2006) hold not only historic events, such as colonization routes, responsible for the low genetic diversity of *R. temporaria* in Sweden, but also current demographic effects, especially small population sizes.

For the taxon *R. esculenta* it always has to be considered that, in spite of low genetic diversity within one genome, the sampled individuals are more diverse, because they are hybrids and, therefore, always heterozygous. Low genetic diversity has only effects when occurring in a homozygous state in a *R. lessonae* (LL) or a *R. ridibunda* (RR) individual; but even then it not necessarily translates into direct fitness consequences. The population near Uppsala is known to have low diversity compared to Central Europe populations (Sjögren 1991b, Sjögren-Gulve and Berg 1999). In these studies, 29 of 31 microsatellite loci (resp. 26 of 28 allozyme loci) were monomorphic. In our study, we found higher diversity for the Uppsala locality, with two out of seven microsatellite loci being polymorphic. But since not the same markers were used, the direct comparison has to be taken cautiously. Despite its low variability, the Uppsala metapopulation of *R. lessonae* seems to do quite well and show no direct loss in fertility or viability (Sjögren 1991a). Similarly, in the Östergötland population, we also detected low genetic diversity in both genomes

(fixed in R and one locus polymorphic in L). In contrast to the pure *R. lessonae* population in Uppsala, we have no long-term data about this population and it is therefore risky to make implications about its viability. However, besides adults, we also sampled many juveniles, suggesting that the population is reproducing successfully.

Colonization and phylogenetic relationships

Neither the mitochondrial DNA nor nuclear DNA yields a clear pattern of colonization for the Swedish populations. It seems that the investigated localities have been connected for a long time and just recently (geologically spoken) split up. Although having a unique haplotype in the mtDNA, the population in Östergötland had no population-specific alleles; all alleles were also found in at least one other population in the sampled region. Based on this result we conclude that these individuals are either native or, if not, have definitely not bee introduced (e.g. food reasons) from France or the Netherlands, as considered possible by earlier reports (J. Pröjts, unpubl.), but come from source populations located in the area under our investigation.

With respect to the mtDNA it was interesting that most of the investigated individuals had *lessonae* mtDNA, except for three animals from Latvia and Lithuania (2 RR/1LR). Most of the sampled individuals were hybrid frogs (diploid and triploids), although two *R. ridibunda* and 8 *R. lessonae* frogs where also among them. For morphological and behavioral reasons it is usually assumed that primary hybridizations took place between *R. ridibunda* females and *R. lessonae* males (Tunner 1974, Berger et al. 1988). Therefore, we would expect to find *ridibunda* mtDNA, since it is inherited maternally (Beebee and Rowe 2004). But if in an LE-system a hybrid lineage goes through at least one mating between a *R. lessonae* female and a *R. esculenta* male, the *lessonae* mtDNA gets subsequently passed on (Spolsky and Uzzell 1986). Although less frequently than expected, these matings (LL♀ x LR♂) occur regularly in LE populations (Abt 2003) and it is therefore not so surprising that we detected *lessonae* mtDNA type in most of our sampled frogs. Even the two *R. ridibunda* frogs, one from Skåne and one from Bornholm, had *lessonae* mtDNA, indicating that they originated from a mixed system where there are (or used to be) *R. lessonae* individuals passing on the *lessonae* mtDNA. Similarly, Hotz et al. (1992) found in an earlier study *R. ridibunda* females with *lessonae* mtDNA in Switzerland and concluded that these females must have resulted from matings between two *R. esculenta* individuals with different hemiclones.

Variability in the mitochondrial DNA was rather small (1.7%), although not unusual for intraspecific variation (Plötner et al. 2001). Our sample consisted of 18 different haplotypes, with one extremely common haplotype (Ht 1a, 44%) and most others present in only one or two individuals. However, there are two additional haplotypes which were found repeatedly; one haplotype (Ht 4, 10%), differing 1 bp from the main haplotype, was endemic to the population in Östergötland (all six individuals) and the second haplotype (Ht 1d, 14%), differing 2 bp from the main haplotype, was only found in eight individuals in Southern Sweden. So, although the phylogenetic tree gives no clear picture from where these frogs colonized the area of Sweden, it can be inferred that both Southern Sweden and Östergötland have been isolated for sufficient time to develop endemic haplotypes. Introduction from Western Europe (i.e. France or the Netherlands) is highly unlikely, since it has been shown that water frogs from Western Europe are genetically very different from the "northern" clade (Hotz et al. 1992, Zeisset and Beebee 2001). Introduction of these Östergötland frogs from the Baltic area can not be completely excluded, but based on mtDNA they did not come directly from any of the populations that we have sampled for this study. From the mtDNA phylogenetic trees and the haplotype network it is difficult to make conclusions about the origin of haplotypes. However, the minimum-spanning network shows that the haplotypes in Central Germany and Italy (3a, 3b, 7a, 7b and 9) are connected, as are the haplotypes from Slovenia, Romania and Poland (10a, 10c and 11). Both these groups are linked with the common haplotype found in the Baltic area (1a). This could indicate that the colonization route came from the Balkan and on one hand went into Northern Italy and on the other hand went up north into Poland, Germany and Denmark similar to what Zeisset and Beebee (2001) suggested for *R. lessonae*.

Unfortunately, the results from the phylogenetic trees based on the microsatellites do not give much more information on a possible colonization for Sweden. In this data set, several populations group together. The frogs from the three Baltic countries are forming one cluster with individuals from two Central Poland populations. This would be consistent with the assumption that the colonization of Estonia, Latvia and Lithuania proceeded from the south through Poland. The remaining populations cluster slightly differently for the two analyses of the different genomes. In the L-genome, we can clearly discriminate two more clusters. One contains the individuals from the Danish island Bornholm, the population near Uppsala, two coastal Polish and two inland German populations. In the other group, we find the Swedish populations with the coastal region of Denmark, Germany and Poland. In the R-genome we, too, find that the Swedish populations

are closest to the coastal population in Germany and some of the Danish populations, but the clustering is less clear. The Östergötland population clusters with the Baltic state populations for the L-genome, whereas for the R-genome the population falls among the coastal German populations. This would support the suture zone hypothesis according to which two lineages meet in Central Sweden. But interestingly, the Uppsala population clusters more with Bornholm than with the Baltic samples, which again argues against the colonization from the north.

For the genetic analyses the two different genomes were treated separately, but it is disputable if this makes biological sense. For the region where only hybrids are found (Northern Germany, Denmark and Southern Sweden) it is apparent that the two genomes would "travel" together. But in regions where parental types are present or even common, it is possible that only on genome migrates, e.g. the L in Baltic LL animals. But since we lack any information about population compositions at the time of post-glacial colonization we are hesitant to draw conclusions about different traveling routes of genomes.

Conclusion

Our intensive sampling of water frogs in the area around the Baltic Sea probably provides a representative overview about the locally existing population systems and the relationship between diversity within a population and its geographical position. Generally, it is assumed that low diversity has a direct negative effects on a population's viability, e.g. through inbreeding. But considering the *R. lessonae* population near Uppsala, this does not seem to always be compulsive. More long-term data on such low diversity populations is needed to disentangle this relationship for the water frogs. Furthermore, we did show in this study that it is difficult to identify a straight forward colonization route into Fennoscandia for this taxon, since no phylogenetic tree structures allowed to distinguish between well established clusters. We interpret this result as follows; first, at the time when postglacial migrations of frogs reached this area, the southern tip of Sweden, Denmark and Germany were all connected (Gislén and Kauri 1959, Lambeck 1995, Snell et al. 2005). Hence, frogs were able to migrate between these areas more easily than we would expect nowadays based on current sea barriers. And second, although these populations must have been connected and in exchange with each other, some areas (as Southern Sweden or Östergötland) have developed endemic haplotypes due to their contemporary isolation. It is in fact these regions that have been cut off first as sea levels were rising.

Acknowledgements

We thank H.-U. Reyer, D. Christiansen, A. Laurila, M. Rybacki, J. Plötner, H. Hotz and G. Guex for providing samples from other localities than Southern Sweden. We are grateful to S. Röthlisberger who helped extracting DNA and Ecogenics for their help with microsatellite analysis of three loci. J. Plötner, F. Köhler, K.-J. Schultze and T. Ohst in Berlin sequenced all the mtDNA samples. Many thanks to M. Bernasconi for his help with the analyses of the mtDNA sequences. This manuscript benefited from the comments of H.-U. Reyer and H. Hotz. This work was supported by Swiss NF grant No. 31-64004.00 to H.-U. Reyer.

Author contributions

M.A. and C.J. contributed equally to this work. Both authors carried out all field- and lab work together. M.A. performed statistical analyses and wrote the paper. Both authors discussed the results and C.J. commented on the manuscript.

References

Abt, G. 2003. Pond use, patterns of reproduction and juvenile recruitment in a mixed waterfrog population. PhD-Thesis. University of Zurich, Switzerland.

Alves, M. J., M. M. Coelho, and M. J. Collares-Pereira. 2001. Evolution in action through hybridisation and polyploidy in an Iberian freshwater fish: a genetic review. Genetica **111**:375-385.

Andersen, B. G., and H. W. Borns. 1997. The ice age world: an introduction to Quaternary history and research. Scandinavian University Press, Oslo.

Babik, W., W. Branicki, M. Sandera, S. Litvinchuk, L. J. Borkin, J. T. Irwin, and J. Rafinski. 2004. Mitochondrial phylogeography of the moor frog, *Rana arvalis*. Molecular Ecology **13**:1469-1480.

Barton, N. H., and G. M. Hewitt. 1985. Analysis of hybrid zones. Annual Review of Ecology and Systematics **16**:113-148.

Beebee, T. J. C., and G. Rowe. 2004. An introduction to molecular ecology. Oxford University Press Inc.

Berger, L. 1967. Embrional and larval development of F_1 generation of green frogs different combinations. Acta Zoologica Cracoviensia **12**:123-160.

Berger, L. 1983. Western Palearctic water frogs (Amphibia, Ranidae): Systematics, genetics and population composition. Experientia **39**:127-234.

Berger, L., and W. A. Berger. 1992. Progeny of water frog populations in central Poland. Amphibia-Reptilia **13**:135-146.

Berger, L., T. Uzzell, and H. Hotz. 1988. Sex determination and sex-ratios in western palearctic water frogs- XX and XY female hybrids in the Pannonian Basin. Proceedings of the Academy of Natural Sciences of Philadelphia **140**: 220-239.

Borkin, L. J., A. V. Korshunov, G. A. Lada, S. N. Litvinchuck, J. M. Rosanov, D. A. Shabanov, and A. I. Zinenko. 2004. Mass occurrence of polyploid green frogs (*Rana esculenta* complex) in eastern Ukraine. Russian Journal of Herpetology **11**:203-222.

Ebendal, T. 1979. Distribution, morphology and taxonomy of the Swedish green frogs (*Rana esculenta* complex). Mitteilungen aus dem Zoologischen Museum Berlin **55**:143-152.

Farris, J. S., M. Källersjö, A. G. Kluge, and C. Bult. 1994. Testing significance of incongruence. Cladistics **10**:315-319.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39**:783-791.

Felsenstein, J. 1993. PHYLIP (Phylogeny inference package) version 3.6 alpha, distributed by the author. Department of Genetics, University of Washington, Seattle.

Fog, K. 1994. Water frogs in Denmark: Population types and biology. Zoologica Poloniae **39**:305-330.

Garner, T. W. J., B. Gautschi, S. Röthlisberger, and H.-U. Reyer. 2000. A set of CA repeat microsatellite markers derived from the pool frog, *Rana lessonae*. Molecular Ecology **9**:2173-2175.

Gislén, T., and H. Kauri. 1959. Zoogeography of the Swedish amphibians and reptiles with notes on their growth and ecology. Acta Vertebratica **1**:196-397.

Günther, R. 1974. Neue Daten zur Verbreitung und Ökologie der Grünfrösche (Anura, Ranidae) in der DDR. Mitteilungen aus dem Museum für Naturkunde Berlin, Zoologische Reihe **50**:287-298.

Günther, R. 1990. Die Wasserfrösche Europas. A. Ziemsen Verlag, Wittenberg.

Günther, R. 1991. Europäische Wasserfrösche (Anura, Ranidae) und biologisches Artkonzept. Mitteilungen aus dem Zoologischen Museum Berlin **67**:39-53.

Günther, R., and J. Plötner. 1990. Mating pattern in pure hybrid populations of water frogs *Rana esculenta* (Anura Ranidae). Alytes **8**:90-98.

Harrison, R. G., and D. M. Rand. 1989. Mosaic hybrid zones and the nature of species boundaries. Pages 111-133 *in* D. Otte and J. A. Endler, editors. Speciation and its consequences. Sinauer Associates, Sunderland, Massachusetts.

Hedges, S. B., J. P. Bogart, and L. R. Maxson. 1992. Ancestry of unisexual salamanders. Nature **356**:708-710.

Hewitt, G. M. 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. Biological Journal of the Linnean Society **58**:247-276.

Hewitt, G. M. 1999. Post-glacial re-colonization of European biota. Biological Journal of the Linnean Society **68**:87-112.

Hewitt, G. M. 2000. The genetic legacy of the Quaternary ice ages. Nature **405**:907-913.

Higgins, D. G., and P. M. Sharp. 1989. Fast and sensitive multiple sequence alignments on a microcomputer. Computer Applications in the Biosciences **5**:151-153.

Holenweg Peter, A.-K., H.-U. Reyer, and G. Abt-Tietje. 2002. Species and sex ratio differences in mixed populations of hybridogenetic water frogs: The influence of pond features. Ecoscience **9**:1-11.

Hotz, H., P. Beerli, and C. Spolsky. 1992. Mitochondrial DNA reveals formation of nonhybrid frogs by natural matings between hemiclonal hybrids. Molecular Biology and Evolution **9**:610-620.

Ibrahim, K. M., R. A. Nichols, and G. M. Hewitt. 1996. Spatial patterns of genetic variation generated by different forms of dispersal during range expansion. Heredity **77**:282-291

Jakob, C. 2007. Structure and dynamics of pure hybridogenetic water frog populations of *Rana esculenta* in Southern Sweden. PhD-Thesis. University of Zurich, Switzerland.

Johansson, M., C. R. Primmer, and J. Merilä. 2006. History vs. current demography: explaining the genetic population structure of the common frog (*Rana temporaria*). Molecular Ecology **15**:975-983.

Kumar, S., K. Tamura, and M. Nei. 2004. MEGA 3: Integrated Software for Molecular Evolutionary Genetics Analysis and Sequence Alignment. Briefings in Bioinformatics **5**:150-163.

Kvindall, O. 1998. Introduktion till sårbarhetsanalyser. ArtDatabanken, SLU, Uppsala.

Lambeck, K. 1995. Late Devensian and Holocene shorelines of the British Isles and North Sea from models of glacio-hydro-isostatic rebound. Journal of the Geological Society **152**:437-448.

Nei, M. 1987. Molecular evolutionary genetics. Columbia University Press, New York.

Okulova, N. M., L. J. Borkin, A. S. Bogdanov, and A. Y. Guseva. 1997. The green frogs in Ivanovo Province. Advances in amphibian research in the Former Soviet Union **2**:71-94.

Palo, J. U., D. S. Schmeller, A. Laurila, C. R. Primmer, S. L. Kuzmin, and J. Merilä. 2004. High degree of population subdivision in a widespread amphibian. Molecular Ecology **13**:2631-2644.

Petit, R. J., I. Aguinagalde, J.-L. de Beaulieu, C. Bittkau, S. Brewer, R. Cheddadi, R. Ennos, S. Fineschi, D. Grivet, M. Lascoux, A. Mohanty, G. Müller-Starck, B. Demesure-Musch, A. Palmé, J. P. Martín, S. Rendell, and G. G. Vendramin. 2003. Glacial refugia: Hotspots but not melting pots of genetic diversity. Science **300**:1563 - 1565.

Plötner, J. 2005. Die westpaläarktischen Wasserfrösche. Laurenti-Verlag, Bielefeld.

Plötner, J., T. Ohst, W. Böhme, and R. Schreiber. 2001. Divergence in mitochondrial DNA of Near Eastern water frogs with special reference to the systematic status of Cypriote and Anatolian populations (Anura, Ranidae). Amphibia-Reptilia **22**:397-412.

Posada, D., and K. A. Crandall. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics **14**:817-818.

Quattro, J. M., J. C. Avise, and R. C. Vrijenhoek. 1992. Mode of origin and sources of genotypic diversity in triploid gynogenetic fish clones (*Poeciliopsis:* Poeciliidae). Genetics **130**:621-628.

Raes, J., and Y. Van de Peer. 1999. ForCon: a software tool for the conversion of sequence alignments. EMBnet.news 6(1). Distributed for free at: http://vulcan.rug.ac.be/~jerae/ForCon/index.html.

Rybacki, M., and L. Berger. 1994. Distribution and ecology of waterfrogs in Poland. Zoologica Poloniae **39**:293-303.

Rybacki, M., and L. Berger. 2001. Types of water frog populations (*Rana esculenta* complex) in Poland. Mitteilungen aus dem Museum für Naturkunde Berlin, Zoologische Reihe **77**:51-57.

Sage, R. D., and J. O. Wolff. 1986. Pleistocene glaciations, fluctuating ranges, and low genetic variability in a large mammal (*Ovis dalli*). Evolution **40**:1092-1095.

SAS Institute. 2002-2003. Version 9.1.3 SP3 for Windows. SAS Institute Inc., Cary, NC.

Schneider, S., D. Roessli, and L. Excoffier. 2000. Arlequin: A software for population genetics data analysis. Ver 2.000. Genetics and Biometry Lab, Dept. of Anthropology, University of Geneva.

Schultz, R. J. 1969. Hybridization, unisexuality, and polyploidy in the teleost *Poeciliopsis* (Poeciliidae) and other vertebrates. The American Naturalist **103**:605-619.

Sjögren, P. 1988. Metapopulation biology of *Rana lessonae* Camerano on the northern periphery of its range. PhD-Thesis. University of Uppsala, Uppsala.

Sjögren, P. 1991a. Extinction and isolation gradients in metapopulations: the case of the pool frog (*Rana lessonae*). Biological Journal of the Linnean Society **42**:135-147.

Sjögren, P. 1991b. Genetic variation in relation to demography of peripheral pool frog populations (*Rana lessonae*). Evolutionary Ecology **5**:248-271.

Sjögren-Gulve, P., and L. M. Berg. 1999. Allozyme variation as a demographic predictor at high latitudes: The moor frog and the pool frog at 600N. Hereditas **130**:317-323.

Snell, C., J. Tetteh, and I. H. Evans. 2005. Phylogeography of the pool frog (*Rana lessonae*) in Europe: evidence for native status in Great Britain and for an unusual postglacial colonization route. Biological Journal of the Linnean Society:41-51.

Söderbäck, O. 1984. Sockna: Bilder från Åtvidaberg. 2:a upplaga edition, Bokugglan i Linköping AB, Linköping.

Spolsky, C., and T. Uzzell. 1986. Evolutionary history of the hybridogenetic hybrid frog *Rana esculenta* as deduced from mtDNA analyses. Molecular Biology and Evolution **3**:44-56.

Swofford, D. L. 2002. PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods). Version 4.0 b10. Sinauer Associates, Sunderland, MA.

Taberlet, P., L. Fumagalli, A.-G. Wust-Saucy, and J.-F. Cosson. 1998. Comparative phylogeography and postglacial colonization routes in Europe. Molecular Ecology **7**:453-464.

Tamura, K., and M. Nei. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution **10**:512-526.

Tunner, H. G. 1974. Die klonale Struktur einer Wasserfroschpopulation. Zeitschrift für zoologische Systematik und Evolutionsforschung **12**:309-314.

Vorburger, C. 2001. Fixation of deleterious mutations in clonal lineages: evidence from hybridogenetic frogs. Evolution **55**:2319-2332.

Vrijenhoek, R. C. 1994. Unisexual fish: Model systems for studying ecology and evolution. Annual Review of Ecology and Systematics **25**:71-96.

Wallis, G. P., and J. W. Arntzen. 1989. Mitochondrial DNA variation in the crested newt superspecies: limited cytoplasmic gene flow among species. Evolution **43**:88-104.

Zeisset, I., and T. J. C. Beebee. 2001. Determination of biogeographical range: an application of molecular phylogeography to the European pool frog *Rana lessonae*. Proceedings of the Royal Society Biological Sciences, Series B **268**:933-938.

Zeisset, I., and T. J. C. Beebee. 2003. Population genetics of a successful invader: the marsh frog *Rana ridibunda* in Britain. Molecular Ecology **12**:639-646.

Zeisset, I., G. Rowe, and T. J. C. Beebee. 2000. Polymerase chain reaction primers for microsatellite loci in the north European water frogs *Rana ridibunda* and *R. lessonae*. Molecular Ecology **9**:1173-1174.

Table 1. The study populations, sample sizes and basic descriptive statistic.

Nr.	Population	Country	Coordinates (N,E)		$N_{\mu sat}$	$N_{ND2\&3}$	H		A	
							L	R	L	R
1	Uppsala	Sweden - SE	60°32'56"	17°53'58"	29	3	0.14 2	n.a.	1.4	n.a.
2	Östergötland	Sweden - SE	58°06'57"	16°24'15"	41	6	0.090	0	1.2	1
3	Skåne 001	Sweden - SE	55°35'17"	13°21'15"	70	2	0. 043	0.131	1.6	2.17
4	Skåne 008	Sweden - SE	55°34'08"	13°19'01"	21	0	0. 037	0.153	1.4	1.67
5	Skåne 014	Sweden - SE	55°34'08"	13°19'01"	24	0	0. 078	0.211	1.6	2.17
6	Skåne 032A	Sweden - SE	55°34'27"	13°13'03"	58	2	0 .100	0.361	1.6	2.50
7	Skåne 050	Sweden - SE	55°29'33"	13°08'02"	38	2	0. 107	0.226	1.6	2.17
8	Skåne 089	Sweden - SE	55°36'34"	13°23'19"	85	2	0. 058	0.098	1.4	1.67
9	Skåne 102	Sweden - SE	55°32'51"	13°17'13"	48	2	0. 135	0.366	1.6	2.67
10	Skåne 138	Sweden - SE	55°31'32"	12°55'45"	54	1	0 .065	0.033	1.4	1.33
11	Skåne 154	Sweden - SE	55°22'24"	13°05'32"	30	3	0	0.203	1	1.83
12	Skåne 159	Sweden - SE	55°22'59"	13°27'01"	30	2	0 .102	0.017	1.2	1.33
13	Bornholm3/4	Denmark - DK	55°08'39"	15°03'42"	22	1	0.062	0.440	1.4	3.50
14	Bornholm 014	Denmark - DK	55°07'23"	15°09'10"	44	0	0.107	0.343	1.4	3.83
15	Bornholm 011	Denmark - DK	55°01'15"	15°01'15"	45	1	0	0.397	1	3.67
16	Bornholm 012	Denmark - DK	55°03'25"	15°00'15"	21	0	0.062	0.492	1.2	3.50
17	N-Seeland 001	Denmark - DK	55°46'14"	12°23'23"	2 4	1	0	0.164	1	1.50
18	N-Seeland 039	Denmark - DK	55°58'08"	12°13'41"	1 2	1	0	0.333	1	1.83
19	S-Seeland 001	Denmark - DK	55°12'08"	11°39'55"	2 7	1	0.092	0.176	1.2	1.67
20	S-Seeland 002	Denmark - DK	55°12'18"	11°29'45"	1 7	1	0.051	0.148	1.2	1.67
21	Ærø	Denmark - DK	54°51'38"	10°23'49"	20	0	0.103	0.212	1.2	1.67
22	Preetz	Germany - DE	54°14'50"	10°11'25"	16	2	0.1 25	0.176	1.6	1.50
23	Fehmarn 011	Germany - DE	54°32'13"	11°03'17"	26	0	0.011	0.256	1.2	1.67
24	Fehmarn 021	Germany - DE	54°29'52"	11°08'45"	23	0	0	0.199	1	2
25	Klützler Winkel	Germany - DE	53°59'29"	11°00'47"	30	0	0.113	0.280	1.4	1.83
26	Hanstorf 07	Germany - DE	54°02'27"	11°54'41"	22	1	0.095	0.275	1.2	2.67
27	Hanstorf 10	Germany - DE	54°02'44"	11°53'58"	34	2	0.105	0.294	1.4	3.17
28	Grammentin 001	Germany - DE	53°41'36"	12°51'11"	26	0	0.111	0.253	1.4	3.17
29	Grammentin 011	Germany - DE	53°41'36"	12°51'11"	14	0	0.083	0.396	1.2	3.17
30	Rügen 011	Germany - DE	54°25'02"	13°23'49"	25	1	0.096	0.315	1.4	2.17
31	Rügen 020	Germany - DE	54°22'48"	13°24'52"	16	1	0.036	0.302	1.2	1.83
32	Usedom	Germany - DE	53°52'45"	14°07'48"	13	0	0.1 51	0.580	1.6	4.00
33	Dargen, Usedom	Germany - DE	53°53'51"	14°03'55"	29	1	0.126	0.554	1.8	4.33
34	Rothemühl 04	Germany - DE	53°34'26"	13°46'04"	29	1	0.265	0.342	2.4	3.00
35	Rothemühl 05	Germany - DE	53°34'19"	13°45'38"	26	1	0.277	0.373	2.6	3.17
36	Swinemünde	Poland - PL	53°52'15"	14°13'28"	19	0	0.179	0.547	1.6	4.50
37	Karsibor	Poland - PL	53°51'07"	14°18'58"	20	0	0	0.620	1	3.83
38	Wiselka	Poland - PL	53°57'39"	14°33'48"	23	0	0	0 .456	1	3.33
39	Kolczewo	Poland - PL	53°57'54"	14°36'35"	9	1	0	0.430	1	2.67
40	Wysoka Kamineska	Poland - PL	53°49'12"	14°50'27"	30	1	0.256	0.515	1. 4	3.50
41	Rogaczewo Wielkie	Poland - PL	52°03'22"	16°49'07 "	44	0	0.198	0.441	1.4	2.67
42	Lubostron	Poland - PL	52°54'33"	17°52'45"	34	0	0 .342	0.599	1.8	5.00
43	Wysoka	Poland - PL	53°10'46"	17°05'08"	20	0	0.16 9	0.466	1.4	4.00
44	Dasunikeskes	Lithuania - LT	54°45'29"	24°08'48"	29	2	0.230	0.561	2	4.33
45	Baltoji Voke	Lithuania - LT	54°35'58"	24°11'56"	33	0	0.232	0.373	2	2.67
46	Grikiapele	Lithuania - LT	55°19'08"	26°02'14"	20	1	0.216	0.306	2.2	1.50
47	Stikli	Latvia - LV	57°18'59"	22°15'54"	33	1	0.30 3	0.089	2.6	1.17
48	Jurmala	Latvia - LV	56°58'00"	23°34'03"	15	3	0.2 00	0.481	1.2	3.33
49	Priedes	Latvia - LV	57°30'38"	25°00'45"	22	0	0.2 38	0.093	2.2	1.33
50	Pärnu	Estonia - ES	58°23'05"	24°31'07"	38	1	0.26 2	0	2	1
51	Laeva	Estonia - ES	58°41'01"	25°35'01"	27	0	0.20 9	n.a.	1.6	n.a.
52	Hara	Estonia - ES	59°05'25"	23°32'17"	21	1	0	n.a .	1	n.a.

$N_{\mu sat}$ = number of individuals genotyped for microsatellites, $N_{ND2\&3}$ = number of individuals sequenced for ND2 and ND3
H = gene diversity, A = average allele number per locus, n.a. indicates that no R-genomes are present in these populations

Table 2. Protocols used for amplification and sequencing of the ND2 and ND3 genes.

	ND2	**ND3**
Double stranded PCR		
Reaction mix (50 µl)	5 µl template DNA (~10 ng) 1.8 U Taq polymerase (InViTek, Berlin, Germany) 5 µl 10x PCR Buffer, Applied Biosystems 2.5 µl $MgCl_2$ (50mM) 0.12 mM each of dATP, dCTP, dGTP, dTTP 1 µM each of primer: Forward L2: 5'-AAGCTTTTGGGCCCATACCCC-3' Reverse H2: 5'-GGGGCGATTTTTTGTCAGGTTG-3' Forward L3: 5'-GGACTCGCCCCYCTACACTTCTG-3' Reverse H3: 5'-CTCCGCTTAAGGCTTTGAAGGC-3'	5 µl template DNA (~10 ng) 1.8 U Taq polymerase (InViTek, Berlin, Germany) 5 µl 10x PCR Buffer, Applied Biosystems 2.5 µl $MgCl_2$ (50mM) 0.12 mM each of dATP, dCTP, dGTP, dTTP 1 µM each of primer: Forward L: 5'-AGTACAAGTGACTTCCAATC-3' Reverse H: 5'-TTGAGCCGAAATCAAATGTC-3'
Amplification PCR profile	1 x (3min at 96℃) 40 x (each of 30s at 96℃, 30s at 62℃, 1min at 72 ℃) 1 x (5min at 72℃)	1 x (3min at 96℃) 40 x (each of 30s at 96℃, 30s at 50℃, 1min at 72℃) 1 x (5min at 72℃)
Sequencing PCR profile	1 x (3min at 96℃) 36 x (each of 15s at 96℃, 15s at 50℃, 4min at 62 ℃)	1 x (3min at 96℃) 36 x (each of 15s at 96℃, 15s at 50℃, 4min at 62℃)

Table 3. Origin (locality, latitude and longitude) and sample sizes (N) of the additional populations that were included in the analysis.

Nr.	Population	Country	Coordinates (N,E)		$N_{ND2\&3}$
1	Carbonare	Italy	45°56'	11°13'	2
2	Caraorman	Romania	45°06'	29°20'	1
3	GheorgheStreamMile	Romania	45°02'	29°10'	1
4	Rogaczewo	Poland	52°04'	16°49'	1
5	Teschendorf †	Germany	52°50'	13°08'	1
6	Mühlenbeck †	Germany	52°51'	13°10'	1
7	OderRiver.Lebus †	Germany	52°25'	14°32'	2
8	VelkeKapusanyVeskovce	Slovakia	48°26'	22°06'	1

†: Sequences of these four individuals contained some insecure sites.

Table 4. Population composition of the 52 populations sampled in Northeast Europe. For population numbers see Table 1. LL = *R. lessonae*, RR = *R. ridibunda*, LR = diploid *R. esculenta*, LLR/LRR = triploid *R. esculenta*.

Population type	Population (see Table 1)
LL	**SE**-1, **ES**-51, -52
LR/LL	**SE**-2, **LT**-45, -46, **LV**-47, -49, **ES**-50
LR/RR	**PL**-37, -39, -42, **LT**-44
LL/RR	**LV**-48
LR/LLR	**DK**-17, **DE**-22, -23, -27, -28, -30, -31
LLR/LRR	**DE**-29
LR/LLR/LRR	**SE**-3, -4, -5, -6, -7, -8, -9, -10, -12, **DK**-14, -15, -18, -19, -20, -21, **DE**-24, -25, -26, -32, -33, **PL**-40, -43
LR/LRR/RR	**SE**-11, **DK**-13, **PL**-38
LR/LLR/LRR/LL	**DE**-34, -35
LR/LLR/LRR/RR	**DK**-16, **PL**-36
LR/LRR/RR/LL	**PL**-41

Table 5. Microsatellite loci used in our study, including their specificity for *R. lessonae* (L-genome) and/or *R. ridibunda* (R-genome).

Microsatellite locus	Specificity	Nr. of L-alleles	Nr. of R-alleles
Ca1b5	L & R	1	3
Ca1b6	L & R	3	8
Re1CAGA10	L & R	2	20
Res16	L & R	2	4
Ga1a19	L & R	1	8
Ca18	L	10	0
Ca5	L	7	0
Re2CAGA3	R	0	16
Average		**3.71**	**9.83**

Table 6. List of haplotypes and population occurrence. 1a is the most common haplotype, occurring in almost all investigated countries (except Poland).

Haplotype	Nr. of individuals	Populations
1a	26	**DK**-15, -17, -18, -19, -20, **SE**-1, -3, -6, -7, -8, -9, -10, -12, **DE**-22, -27, -30, -31, -33, -34, -35, **ES**-50, **LV**-48, **LT**-46, **RO-**Caraorman
1b	1	**LV**-47
1c	2	**PL**-39, -40
1d	8	**SE**-3, -6, -7, -8, -9, -11
2	2	**DE**-26, -27
3a	1	**DE**- Mühlenbeck
3b	1	**DE**- Teschendorf
4	6	**SE**-2
5	1	**DK**-13
6	1	**SE**-1
7a	1	**DE**- OderRiver Lebus
7b	1	**DE**- OderRiver Lebus
8	1	**ES**-52
9	2	**IT-**Carbonare
10a	1	**RO-**Gheorghe Stream Mile
10b	1	**DE**-22
10c	2	**LT**-44, **PL-**Rogaczewo
11	1	**SK-**Velke Kapusany Veskovce

Figure 1. Distribution map for *Rana lessonae*, *Rana ridibunda* and their hybrid *Rana esculenta*.

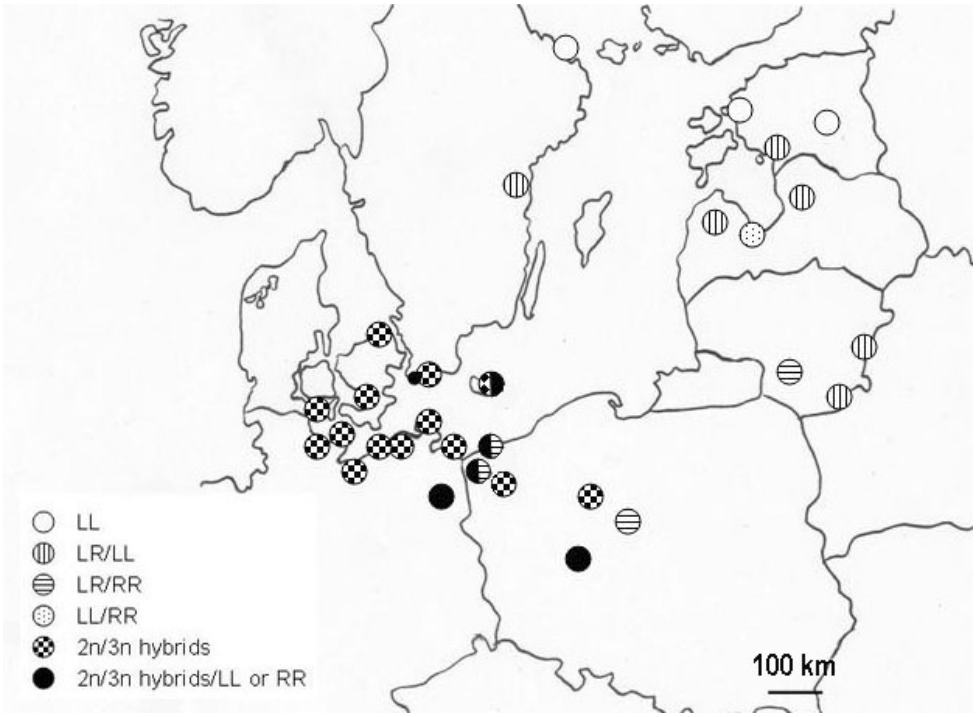

Figure 2. Compositions of the investigated populations located around the Baltic Sea. Dots represent one to nine (Southern Sweden) different populations sampled in the same area.

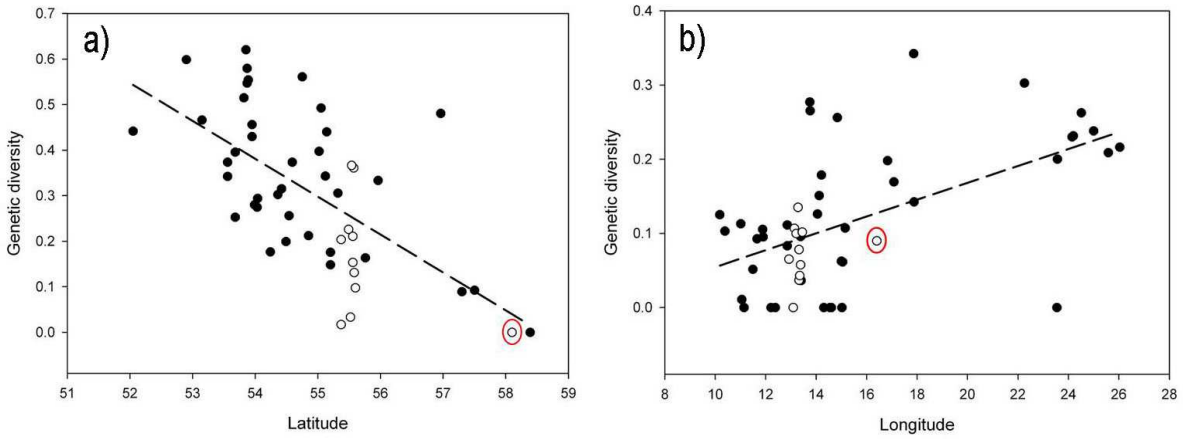

Figure 3. Relationship between (a) latitude and genetic diversity in the R-genome and (b) longitude and genetic diversity in the L-genome. The most southern population is Rogaczewo in Poland; the most northern population is near Uppsala in Sweden. The most western population is Preetz in Germany and the most eastern population is Grikiapele in Lithuania. Open circles are populations in Skåne; the encircled point denotes the population in Östergötland.

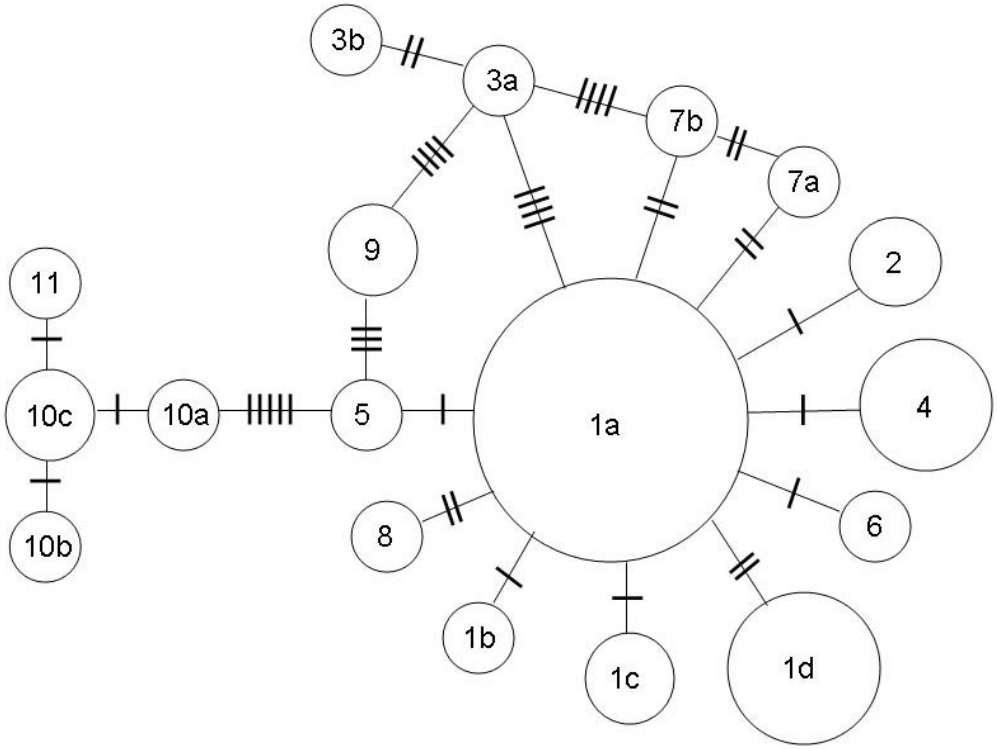

Figure 4. Minimum-spanning network relating the *lessonae* mtDNA haplotypes in waterfrogs. Numbers indicate haplotypes that differ in the amino acid sequence. Letters indicate sub-haplotypes which differ only in synonymous substitutions. Small dashes show the number of substitutions from one haplotype to the other. Size of the circles represents the number of individuals having this haplotype.

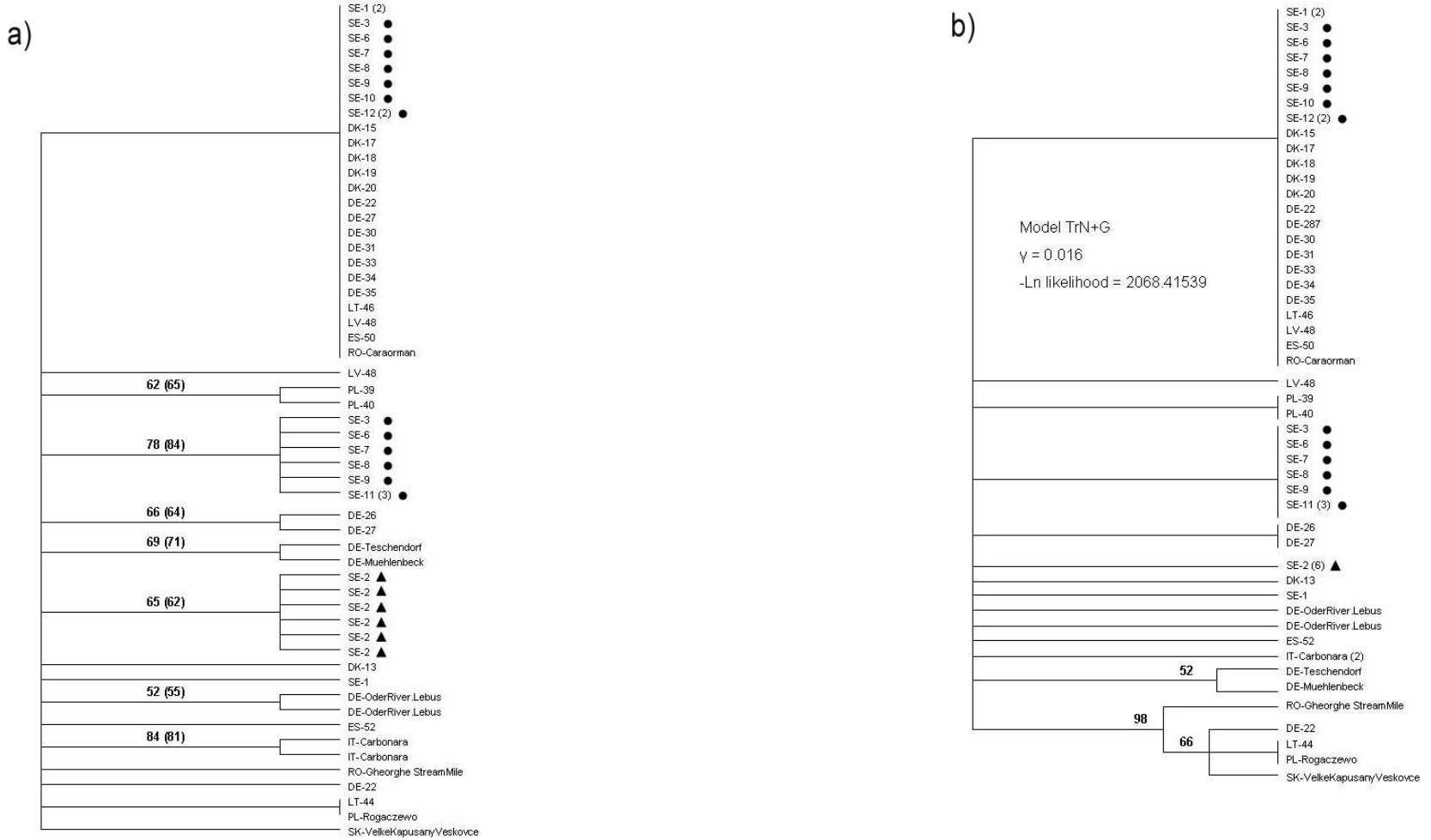

Figure 5. Maximum parsimony and neighbour-joining (a) and maximum likelihood (b) trees for 59 individuals (*lessonae* mtDNA). Numbers indicate bootstrap values, those in brackets are values from the NJ method. ● indicates individuals from Southern Sweden, ▲ depicts frogs from Östergötland.

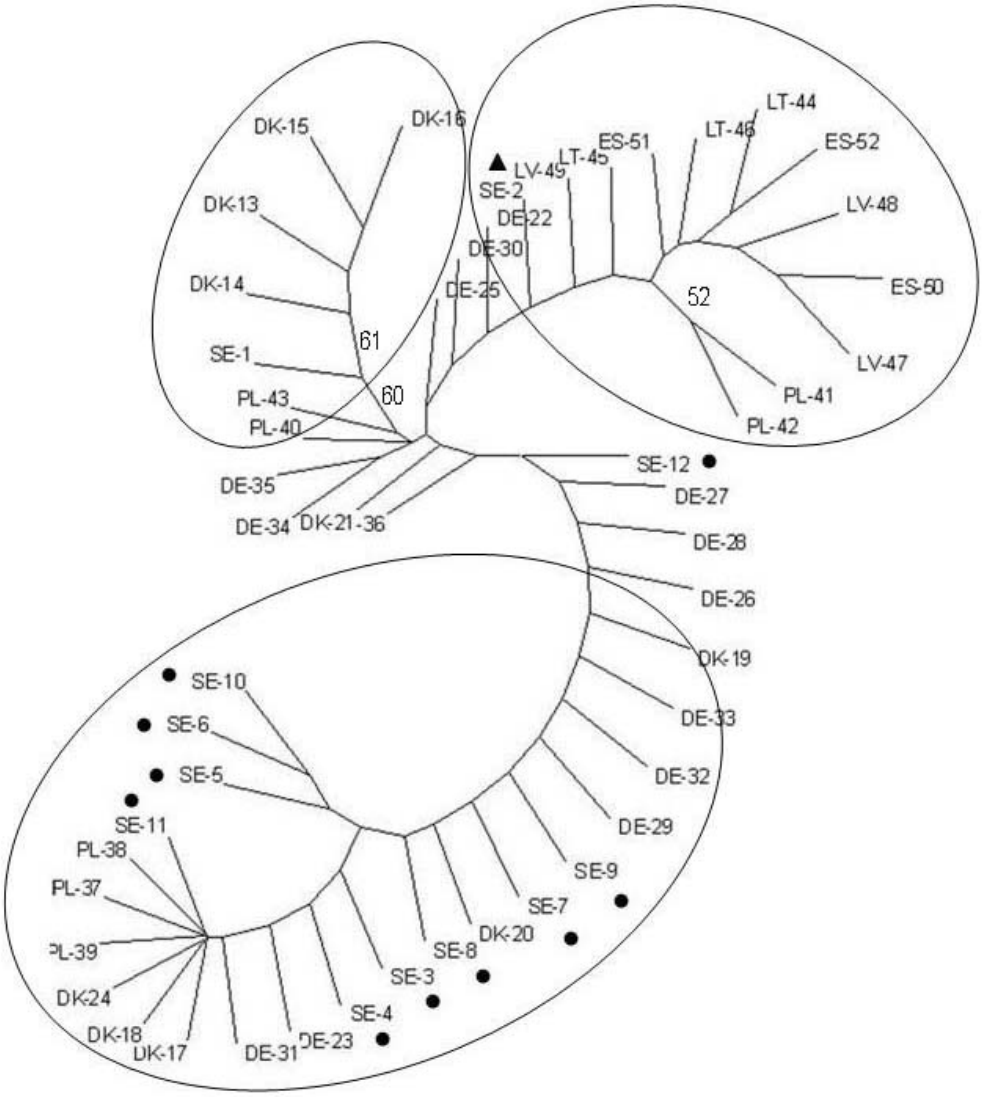

Figure 6. Unrooted NJ tree based on 52 different populations for the L-genome. Population names correspond to Table 1. ● indicates individuals from Southern Sweden, ▲ depicts frogs from Östergötland. Bootstrap support values > 50 indicated by the nodes. Encircled are rough clusters.

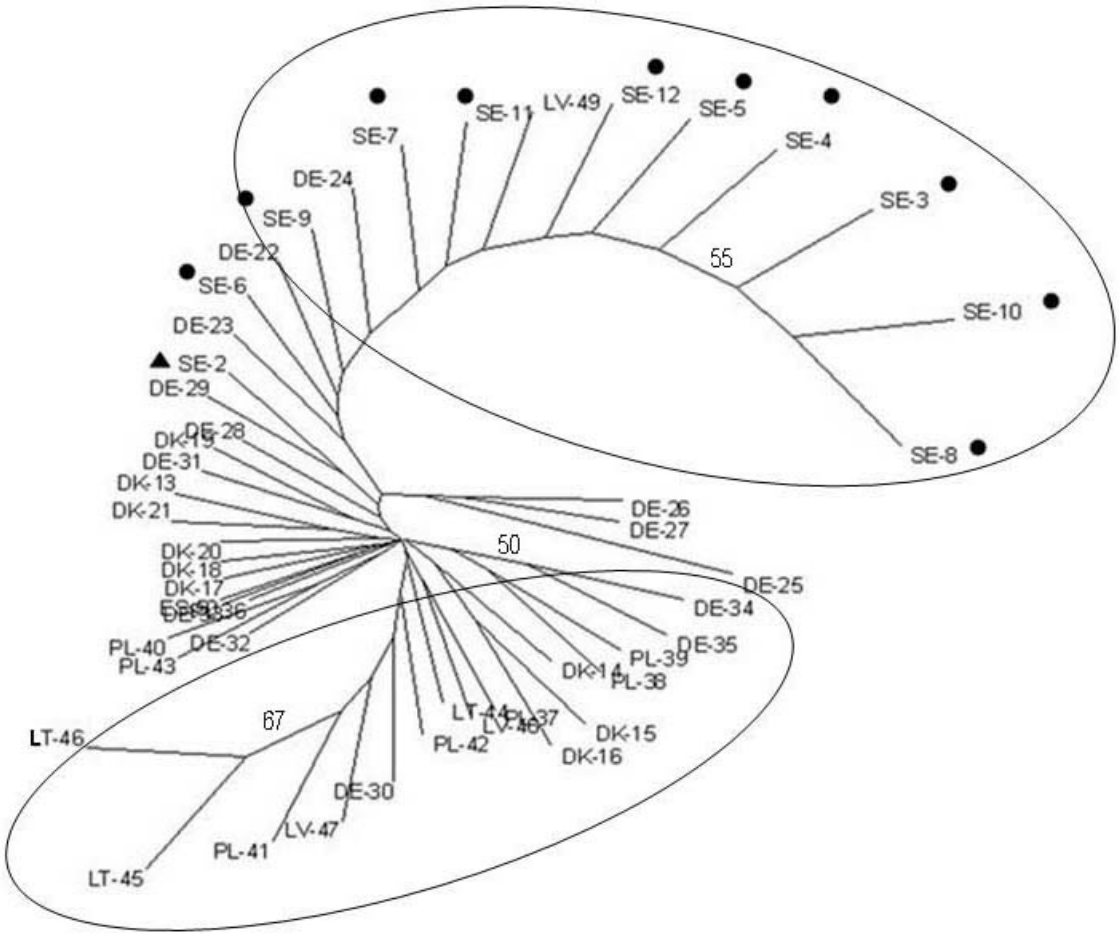

Figure 7. Unrooted NJ tree based on 49 different populations for the R-genome. Population names correspond to Table 1. ● indicates individuals from Southern Sweden, ▲ depicts frogs from Östergötland Bootstrap support values > 50 indicated by the nodes. Encircled are rough clusters.

ACKNOWLEDGEMENTS

First of all, I would like to thank Uli Reyer for giving me the opportunity for this thesis, his scientific and financial support and help with all the little difficulties arising when conducting fieldwork abroad. I also warmly thank the following people who helped me in various ways during my thesis: Sandy Röthlisberger for her endless patience in the lab while cutting frog toes and for the enjoyable coffee breaks, Anni Mäder for all her help with administrative things, Ditte Christiansen, Eline Embrechts, Trent Gurner and Ursina Tobler for their help in the field, Hansjürg Hotz and Gaston Guex for sharing knowledge about the small green creatures, Annette Sauter, Benedikt Schmidt, Christoph Sandrock, Christoph Vorburger, Karin Schiegg, Gilberto Pasinelli, Peter Wandeler and Marco Bernasconi for all kinds of interaction and many other people in the ecology lab.

Thanks also to Kathrin Tschannen and Katja Fink of the group of R. Zinkernagel and especially Eva Niederer from the IBT who made the myriad hours of blood analysis bearable.

The time in Sweden was extraordinary and memorable: Jon Loman, Bodil Enoksson and Torkel welcomed us warmly every year and introduced us to the Swedish frogs, delicious BBQs and blue tadpole tanks, Jan Pröjts took as along to the north and kindly shared his knowledge about the Swedish herpetofauna, Henrik Smith and Gunilla Lindquist facilitated our stay at Stensoffa, Bo Baldetorp and Maria Johansson introduced us patiently to flowcytometry. Many people passed through the field station during these three summers and brightened our days in Sweden, especially Rachel Muheim, Jean Secondi, Stefan Gödderz and Michi Tobler.

Finally, but most importantly I thank my family and friends. You gave me moral support when it was most needed during rough times of my thesis. My heartfelt thanks to Christian Jakob, without whom this thesis would not have been possible, jag älskar dig oändligt!

CURRICULUM VITAE

Personal

Name	Martina Arioli
Birth	19. September 1975, in Bülach ZH
Citizenship	Swiss, Gurtnellen UR

Education

2002 - 2007	Dissertation at the Institute of Zoology, University of Zürich: “Reproductive patterns and population genetics in pure hybridogenetic water frog populations of *Rana esculenta*“, under the supervision of Prof. H.-U. Reyer, University of Zürich.
1998 - 1999	Diploma Thesis at the Institute of Zoology, University of Zürich: „Adaptation of *Rana temporaria* populations to predator regime“, under the supervision of Dr. Josh Van Buskirk, University of Zürich.
1995 – 2000	Studies of biology at the University of Zürich. Main subject: zoology (ecology, behavioral biology, taxonomy and morphology). Second subject: systematic botany.
1990 – 1995	Kantonsschule im Lee, Winterthur, Matura, Typus C.

Publications

Van Buskirk, J. and M. Arioli. 2002. Dosage response of an induced defense: how sensitive are tadpoles to predation risk. Ecology 83: 1580-1585.

Van Buskirk, J. and M. Arioli. 2005. Habitat specialization and adaptive phenotypic divergence of anuran populations. Journal of Evolutionary Biology 18:596-608.